# Pig Oocyte Activation and Developmental Competence of Parthenogenetically Activated Oocytes: *In vitro* and *In vivo* studies

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# Declaration

I declare that this thesis has been completed by myself and has not been submitted for any previous degree. The work described herein was done by myself and all work that was done by others is duly acknowledged. Also, I would like to acknowledge all helps given to me during the course of these studies.

Jie Zhu

# Abstract

In somatic cell nuclear transfer in mammals, to clone a piglet is still a big challenge. Although many factors could contribute to the low success rate, such as quality of donor and recipient cells, type of donor cell including sources of animal breeds and tissues, number of passages and culture conditions, timing of cell cycle, procedures of nuclear transfer, techniques and the number of survival cloned embryos, embryos transfer, one of these factors is believed to be poor oocyte activation, especially in pig nuclear transfer. Therefore studies presented in this thesis aimed at the establishment of an *in vitro* culture system for pig oocyte maturation and embryo culture, based on this system an electrical activation protocol for pig oocytes was optimized and also tested by monitoring *in vivo* development of activated pig oocytes. Finally, the protocol was used for activating pig embryos reconstructed by transfer of somatic cells into enucleated ovulated oocytes and for production of pig parthenotes to maintain pregnancies of cloned pig embryos, which resulted in the birth of a cloned male piglet.

The thesis comprises a total of 6 chapters. In addition to the review of literature (Chapter 1), general materials and methods (Chapter 2) and general discussion (Chapter 6), in Chapter 3 and 4, the studies focused on optimizing electrical parameters on pig oocyte activation and investigating the effects of activation conditions including temperature, activation medium, and concentrations of $Ca^{2+}$ and $Mg^{2+}$ in activation medium and diploidization of activated oocytes. These experiments were carried out *in vitro*, whereas experiments in Chapter 5 were

conducted *in vivo* to assess the *in vivo* developmental competence of *in vitro* matured (IVM) pig oocytes activated by the improved activation protocol.

In Chapter 3, pig oocytes were matured in NCSU 23 medium + 10% pig follicular fluid (pFF) (1); NCSU 23 medium + 10% pFF + amino acids (essential and non essential) (2); NCSU 23 medium + pFF + 10μg/ml epidermal growth factor (EGF) (3) and NCSU 23 medium + pFF + amino acids + EGF (4) for 44 h. The medium (2) yielded the highest blastocyst rate of activated oocytes, which was significantly higher than those in the medium (3) and (4) ($p<0.05$). Oocytes matured in medium (2) resulted in a maturation rate of about 75% at 36 h of maturation, afterwards the rate reached over 90%. Subsequently, four experiments were carried out to examine the interactions among age of oocytes, electrical field strength, pulse number and duration. These results demonstrate that the best stimuli are 3 x 80 μsec consecutive pulses of 1.0 kV/cm DC using 44 h post matured oocytes, and suggest that using 3 consecutive pulses of low electrical field strength for shorter duration is beneficial for activating oocytes and developmental potential.

In Chapter 4, based on the improved electrical activation protocol the effects of activation conditions were examined including activation temperature, activation media, and $Ca^{2+}$ and $Mg^{2+}$ concentrations in activation medium as well as diploidization of the activated oocytes. The results reveal that temperature; $Ca^{2+}$ but not $Mg^{2+}$ concentration in activation medium and diploidization are very important factors for successful oocyte activation. The blastocyst rate of activated pig oocytes was highly correlated with the changes of temperature ($R = 0.97$; $p<0.01$). Similarly, the percentages of diploid blastocysts were also correlated with either the proportions of diploid oocytes or the blastocyst rates ($R=0.89$; $p<0.05$ and $R= 0.959$;

$p<0.05$). Moreover, the results suggest that the minimum required time of cytochalasin B treatment to the activated IVM pig oocytes is 3 hours for achieving a reasonable blastocyst rate with over 70% of activated oocytes being diploid.

In Chapter 5, IVM and ovulated pig oocytes were electrically activated and transferred into synchronous recipients in order to assess their *in vivo* developmental potential. The pregnancies of the transferred IVM pig parthenotes were not detected by ultrasound examination between 40-50 days of gestation. Pig parthenotes were surgically collected from 11 pregnancies on day 21, 30 and 35 of gestation, respectively. These results showed that either ovulated or IVM pig oocytes following activation with the protocol were able to develop *in vivo* for 30 days, suggesting that they stopped development around day 31 of gestation. Although these parthenotes were smaller and lighter, majority of them were morphologically normal. In addition, transferring the reduced number of day 2 cleaved oocytes into recipient animals, compared with the overnight cultured oocytes, seemed not to affect the pregnancy rate (85%; 11/13 vs. 75%; 3/4).

# Publications arising from the studies in the thesis

**Jie Zhu,** Evelyn E Telfer, Judy Flectcher, Anthea Springbett, John Dobrinsky, Paul DeSousa and Ian Wilmut. (2001) Improvement of electrical activation protocol for porcine oocytes. *Biology of Reproduction* (accepted)

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Paul DeSousa, Timothy J. King, **Jie Zhu,** Alan Archibald Andras Dinnyes, John Dobrinsky, Ian Wilmut (2001) Pig cloning by somatic cell nuclear transfer. *Sixth International Conference on Pig Reproduction* Abst. 145

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1999 the second prize of PhD students' poster day at the Roslin Institute. Scotland

Co-inventor on the US patent: Porcine Oocytes with improved Developmental Competence" (09/832,312)

# Abbreviations

| | |
|---|---|
| BSA | Bovine Serum Albumin |
| COCs | Cumulus Oocyte Complexes |
| CB | Cytochalasin B |
| CH | Cycloheximide |
| AA | Amino Acids |
| EGF | Epidermal Growth Factor |
| IGF2 | Insulin like Growth Factor 2 |
| pFF | Porcine Follicular Fluid |
| NCSU 23 | North Carolina State University23 Medium |
| DC | Direct Current |
| AC | Alternating current |
| eCG | Equine Chorionic Gonadotrophin |
| hCG | Human Chorionic Gonadotrophin |
| LH | Luteinising hormone |
| IVM | *In Vitro* Maturation |
| GV | Germinal Vesicle |
| MI | Metaphase I |
| MII | Metaphase II |
| $CO_2$ | Carbon Dioxide |
| $Ca^{2+}$ | Calcium Ions |
| $Mg^{2+}$ | Magnesium Ions |
| $Sr^{2+}$ | Strontium Ions |
| $InP_3$ | Inositol 1,4,5-trisphosphate |
| Min | Minute(s) |
| H | Hour(s) |
| µsec. | Microseconds |
| sec. | Second(s) |
| mm | Millimetre(s) |
| Kg | Kilogram(s) |
| ml | Millilitre(s) |
| µl | Microlitre(s) |
| µm | Micrometer(s) |
| °C | Centigrade(s) |
| µg | Microgram(s) |
| mM | Millimolar (s) |
| UV | Ultraviolet |
| ICSI | Intracytoplasmic Sperm Injection Procedure |
| BSE | Bovine Spongiform Encephalitis |

| | |
|---|---|
| GVBD | Germinal Vesicle Break Down |
| IVF | *In Vitro* Fertilisation |
| MPF | Maturation / Mitogen Promoting Factor |
| DNA | Deoxyribonucleic Acid |
| DMSO | Dimethyl Sulphoxide |
| 6-DMAP | 6-Dimethylaminopurine |
| MAP | Mitogen–Activated Protein |

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# Chapter 1

# Review of Literature

## 1.1. Introduction

The history of animal nuclear transfer dates back to the early 1990's, the concept of nuclear transplantation was first proposed by Speman (1938) who assumed that all the nuclei within an early embryo are genetically identical and each could be grafted to an enucleated recipient oocyte. This would result in the possibility of a large number of identical single cell embryos. Nuclear transfer for the study of differentiation in amphibians has been used since the 1950's (Prather and First, 1990). However, the cloning of mammals really started with the development of nuclear transfer methods in the mid-to-late 1970s (Solter, 2000). Before Dolly, a sheep cloned from a adult somatic cell (Wilmut *et al,* 1997), the cloning of mammals from embryonic cells had been achieved in several species, such as mice (Illmensee and Hoppe, 1981), rabbits (Bromhall, 1975), sheep (Willadsen., 1986; Smith and Wilmut, 1989), pigs (Prather *et al.*, 1989), goats (Zhang *et al.*, 1991), cattle (Westhusin *et al.*, 1991; Bodioli, 1993; Stice *et al.*, 1994). However, in most of these successes, donor cells were from 4 and 8 cell blastomere nuclei. In 1995 two lambs that were cloned from cultured embryonic cells originally derived from a nine-day-old embryo were born (Campbell *et al.,* 1996) and subsequently, the first sheep cloned from an adult cell was born in 1996 (Wilmut *et al.,* 1997), thus demonstrating that cloning of adult mammals by nuclear transfer was possible. Since the birth of Dolly, animal cloning by nuclear transfer using somatic cells as donor cells has been developing dramatically. To date cloned animals resulting from

somatic cell nuclear transplantation have been reported in mice (Wakayama *et al.*, 1998), cattle (Cibelli *et al.,* 1998), goats (Baguisi *et al.*, 1999), sheep (Wilmut *et al.*, 1997), and more recently pigs (Polejaeva *et al.*, 2000). Work involving other species is currently ongoing, and information gathered to date suggests a wide variety of different animal species can be cloned by nuclear transplantation (Westhusin *et al.*, 2001). Although different protocols of nuclear transplantation in mammals have been used worldwide, cloning animals by nuclear transplantation involves similar key steps including; (1) collection of mature oocytes, (2) removing chromosomes from the oocyte (enucleation), (3) transfer of cell nuclei derived from the animal into the enucleated oocyte, (4) fusion of recipient oocyte and the donor cell or injection of the donor cell into an enucleated oocytes, (5) activation of the reconstructed embryo, (6) culture of the embryos, and (7) transfer of the cloned embryo into a synchronous animal.

Cloning by nuclear transfer has huge, potential benefits to agriculture, cell biological research, cell therapy and medical research including human xenotransplantation. Shortly after the birth of Dolly, human embryonic stem cells were achieved by Thomson *et al* (1998), which showed a coming of age of cell technology. Subsequently, several exciting results from published experiments (Flax *et al.*, 1998; Brüstle *et al.*, 1998; Zawada *et al.*, 1998; Bjornson *et al.*, 1999) with stem cells demonstrated that large quantities of disease free cells might be produced for transplants, such as pancreatic beta cells for the treatment of diabetes, neuronal implants for Parkinson's disease, or cells for brain, nerve, and heart graft. The concept of using embryonic stem cells as the starting materials for producing therapeutically useful populations of

differentiated cell types or their stem cell precursors for transplantation purposes was described in 1980 (Edwards and Steptoe, 1980). The advent of somatic nuclear transfer has led to a refinement of this concept with the prospect of embryonic stem cells being derived from embryos reconstructed using a nucleus taken from a healthy cell from the afflicted patient (Trounson and Pera, 1998); in this way the immunological problems associated with allografting could be mostly avoided, although small differences may arise because mitochondria that are exclusively derived from the oocyte in somatic nuclear transfer (Evans *et al.,* 1999) encode minor hisocompatiblity antigens. Therefore, the combination of human stem cell and somatic nuclear transfer technology has become a potential powerful tool to treat some human diseases that were thought to be untreatable. Additionally, nuclear transfer is an alternative to pronuclear microinjection to create transgenic animals. Compared to pronuclear microinjection, nuclear transfer has several advantages in creating transgenic animals (Wells *et al.*, 1999) (Table1.1.). The biggest advantage is that gene modification such as gene transfer or targeting can be carried out on cultured cell lines that will be used as donor cells so that we can select cell lines whose desired gene(s) has been modified as well as the sex of the cell lines before nuclear transfer. This will enable 100% of cloned offspring to have exactly the same desired gene modification and the expected sex, whereas only 1-5% of offspring created by the pronuclear microinjection would be transgenic and their sex is unpredictable (Wells *et al.*, 1999). In addition to cell therapy, nuclear transfer would also be beneficial for improvement of meat/milk production, creating disease resistant agricultural animals such as BSE free animals, using agricultural animals as human

disease models such as cystic fibrosis sheep model, biopharmaceutical protein production such as α-1-antitrypsin and α-glucosidase, and human xenotransplantation. Moreover, using identical animals produced by nuclear transfer would be the best way to enable meaningful results using the minimum number of animals in genetic, medical and animal nutrition studies. Taken together, nuclear transfer, especially somatic cell nuclear transfer provides a powerful tool in a wide range of areas.

**Table 1.1. Characteristics of two techniques for producing transgenic animals.**

| ASPECT | PRONUCLEAR MICROINJECTION | SOMATIC CELL NUCLEAR TRANSFER |
|---|---|---|
| | | |
| Vector preparation difficulty* | Low | Moderate |
| Transgene size limit | ≈ Infinite | ≈ Infinite |
| Gene targeting | Might be possible | Possible |
| Embryo manipulation skill required | High | High |
| Embryo survival | Low to moderate | Moderate |
| Fetal survival | Moderate | Low |
| Proportion born transgenic | 1-10% | 100% |
| Occurrence of mosaicism | Moderate | Zero |
| Number of transgene copies | High | Selectable |
| Multiple insertions | Low | Low |
| Expressing founders | 50% | 50%§ |
| Expressing progeny | ≈ 100% | ≈ 100% |

* Includes DNA preparation and other procedures required before embryo manipulation.
§ No data available but expected to be the same as pronuclear microinjection.
The table is cited from Kevin Wells *et al* (1999). Nature Biotechnology 17: 25-26

**Table 1.2. Cloning efficiency**

| SPECIES | NUCLEAR DONOR CELL TYPE | NUMBER OF OOCYTES MANIPULATED | LIVE BIRTHS /ADULTS (%)* |
|---|---|---|---|
| Sheep | Embryonic epithelium Campbell K, *et al* (1996) | 244 | 5/2(0.8) |
| | Mammary epithelium Wilmut, I *et al* (1997) | 277 | 1/1(0.4) |
| | Fetal fibroblasts TR Schnieke, AE *et al* (1997) | 507 | 6/4(0.8) |
| | Fetal fibroblasts TR McCreath, K J *et al* (2000) | 417 | 14/3(0.7) |
| | | | |
| Cow | Cumulus cells Kato., Y. *et al* (1998) | 99 | 5/2(2.0) |
| | Oviductal cells Kato., Y. *et al* (1998) | 150 | 3/2(1.3) |
| | Fetal fibroblasts TR Cibelli, J. B *et al* (1998) | 276 | 4/3(1.1) |
| | Adult fibroblasts Kubota, C. *et al* (2000) | 1,103 | 6/4(0.4) |
| | Senescent fibroblasts Lanza, R. P *et al* (2000) | 1,896 | 6/6(0.3) |
| | | | |
| Mouse | Cumulus cells Wakayama, T *et al.* (1998) | 1,345 | 16/10(0.7) |
| | Adult fibroblasts Wakayama, T and Yanagimachi, R (1999) | 717 | 3/1(0.4) |
| | ES cells Wakayama, T. et al (1999) | 1,765 | 5/1(0.05) |
| | ES cells$\phi$ F1 Wakayama, T. et al (1999) | 1,087 | 26/13(1.2) |
| | ES cells Rideout, W.M *et al* (2000) | 418 | 8/0 |
| | ES cells F1 Rideout, W.M *et al* (2000) | 227 | 7/7(3) |
| | | | |
| Pig | Fetal fibroblasts Onishi, A *et al* (2000) | 110** | 1/1(0.9)*** |
| | Adult granulosa cells Polejaeva, I.A et al (2000) | 401** | 5/5(1.2)*** |

* represents clones surviving to adulthood as a percentage of total number of manipulated oocytes. $\phi$ Nuclei were derived from an R1 cell line. ** Number of embryos actually transferred into surrogate sows. Number of manipulated oocytes was 2-3 times higher. *** Calculated on the basis of embryos transferred. (ES, embryonic stem; TR, transgenic) The table is cited from "Mammalian Cloning; Advances and limitations" written by Davor Solter (Nature Reviews Genetics 2000: 199-207)

Although nuclear transfer techniques have been developed rapidly and success of nuclear transfer in sheep, mice, cattle, goats and pigs with somatic cells have been reported, the efficiency of somatic nuclear transfer is still extremely low (<1%) (Table1.2).

During normal fertilisation, sperm causes oocyte activation otherwise the oocyte will die within 48 h. However, there is no sperm contribution to a nuclear transfer embryo, so activation in the nuclear transfer embryo must be carried out artificially and the efficiency of this success may contribute to the success of nuclear transfer.

Following the success in sheep, successful nuclear transfer was reported in mice (Wakayama et *al.,* 1998) and cattle (Kato *et al.*, 1998) but the biggest challenge was to clone a pig since several hurdles including activation and maintenance of pig pregnancy had to be overcome. This project "pig oocyte activation" was initially aimed at improving the efficiency of pig oocyte activation (parthenogenetic activation). At that time, the efficiency of pig oocyte activation according to the publications was very low compared to those of sheep and cattle (Wang *et al.*, 1998). Even now, three groups announced that they successfully cloned pigs. These results on oocyte activation were still poor (Onishi *et al.,* 2000; Betthauser *et al.,* 2000). For example, the PPL Company first successfully cloned pigs with a serial nuclear transfer, namely twice nuclear transfers. Enucleated oocytes and enucleated zygotes were used as recipient cells for the first round and second round of nuclear transfer, respectively. The reason for the use of the serial nuclear transfer was because of system that uses fertilised zygotes as cytoplast recipients would bypass the inefficiencies of artificial activation procedures and might

promote more successfully development (Polejaeva *et al.*, 2000). It is clear that poor oocyte activation in this case had been bypassed rather than been resolved. The second success of pig nuclear transfer with electrical activation showed that blastocyst rate of ovulated and activated pig oocytes was 31.2% (34/109), compared with only 2.4% (14/167) when using *in vitro* matured (IVM) oocytes (Onishi *et al.*, 2000). More recently, piglets were cloned with *in vitro* matured oocytes as recipient cells (Betthauser *et al.*, 2000). However, the data showed that blastocyst rate of only 23% (235/1028) was obtained by activating IVM sow oocytes with ionomycin (Betthauser *et al.*, 2000). These results prove that poor oocyte activation may contribute to the inefficiency of pig nuclear transfer.

## 1.2. *In Vitro* Maturation of Porcine Oocyte (IVM)

*In vitro* maturation of pig oocyte means that immature pig oocytes that are obtained from ovarian follicles can be cultured in a laboratory medium to allow the completion of cellular events that enable fertilisation and full developmental competence. Oocyte maturation is the transformation of an oocyte into a fertilisable egg, occurring upon cessation of the first prophase arrest and progression through metaphase I to the second metaphase of meiosis. Oocyte maturation can be divided into two different stages: nuclear maturation and cytoplasmic, maturation. Yamauchi *et al* (1996) showed that culture conditions used for the culture of porcine oocytes *in vitro* are important with respect to their subsequent response to artificial activation.

Therefore, the establishment of an effective maturation system for pig oocytes is essential to improve pig oocyte activation.

### 1.2.1. Effect of follicle size and selection of oocytes maturation media

The size of follicle that the oocyte is derived from has an effect on IVM success. Motlik *et al* (1984) isolated pig oocytes from follicles 0.3-0.7, 0.8-1.6, 1.7-2.2 and 3-5 mm in diameter, respectively, and cultured then for 48 h. The results showed that after 24 h of culture, more than 80% of oocytes from follicle 0.3-0.7 mm in diameter remained at the germinal vesicle stage, whereas 66, 94.3 and 100% oocytes from the other groups, respectively, completed germinal vesicle breakdown. After 48 h of culture, 35% of the oocytes in the smallest follicle class progressed to pro-metaphase I and only 4% to metaphase I. Of the oocytes from follicles 0.8–1.6 mm in diameter, 23% reach metaphase I and 17.3% metaphase II. About 50 and 76% of the oocytes from follicles 1.8–2.2 mm and 3-5 mm in diameter, respectively, extruded the first polar body. Furthermore, the ability to complete meiotic maturation is acquired in antral follicles of about 2 mm in diameter and this coincides with a significant decrease in the nuclear transcriptional activity. A high proportion of oocytes from small antral follicles (1.0-2.0 mm) have been found to be atretic (McGaughey *et al.*, 1985). Hirao, *et al* (1994) reported that the relationship between the size and meiotic competence was similar for oocytes grown *in vitro* or *in vivo*. According to these data, it could be concluded that the pig cumulus-oocyte complexes (COCs) should be taken from antral follicles at least bigger than 2 mm in diameter for successful maturation *in vitro*.

It has been confirmed that cumulus cells play an important role during *in vitro* maturation of pig oocytes. Ocampo *et al* (1994) reported significant differences between nuclear maturation and male pronuclear formation of oocytes matured with cumulus cells and those without or lacking cumulus cells. The rate of nuclear maturation and the rate of male pronuclear formation in oocytes with or without cumulus cells were over 90% or below 66% and less than 40%, or near 80%, respectively, when the oocytes were cultured in a modified TCM 199 medium supplemented eCG and hCG for 42 h. Petr *et al* (1989) cultured 10 pig COCs in 10 micro-litre droplets of culture medium for 24 h, germinal vesicle breakdown (GVBD) occurred in 81% of them. However, when more COCs (20 or 40) were cultured in the same volume of the medium the frequency of GVBD gradually decreased, whereas this inhibition was not observed in denuded oocytes. Furthermore, It has been demonstrated that oocytes were cultured for longer with cumulus cells during maturation *in vitro*, show a higher rate of penetrability (Mattioli *et al,* 1988). Yet, Wang *et al* (1997) reported that cumulus cells mediate the role(s) of both EGF and gonadotropins in stimulating cytoplasmic maturation of pig oocytes, because neither EGF nor gonadotropins exhibited a stimulatory effect on male pronuclear formation in cumulus-free oocytes. This is evidence that cumulus cells are involved not only in controlling nuclear maturation, but also in synchronising nuclear and cytoplasmic maturation.

Nuclear maturation in pigs was described by Edwards in 1965 and proceeded to the metaphase II stage *in vitro* within 46 h. Pig oocytes require almost twice the time to transform from the prophase nucleus, the germinal vesicle, into condensing chromatin

(normally 44-48 h) compared to other domestic mammals (20-26 h in sheep and cows), therefore ensuring correct maturation media is essential. Tissue culture medium (TCM)-199, a common medium for oocyte maturation in most species, has been used in most laboratories (Mattioli *et al* 1988; Yoshida *et al.,* 1990; Wang *et al.,* 1991; Funahashi and Dave, 1993; Funahashi *et al*, 1994 a, b). Blastocyst development (Mattioli *et al.,* 1988; Yoshida *et al.*, 1993; Funahashi *et al.,* 1994a, b) and birth of piglets (Mattioli *et al.*, 1989) resulting from oocytes matured in TCM-199 have been reported, although the efficiency is very low. A modified Whitten's medium (mWM) (Funahashi *et al.,* 1994a, b) and a bovine serum albumin (BSA)-free NCSU-23 medium (Funahashi *et al.,* 1996), which were primarily developed for the culture of pig preimplantation embryos (Petters and Wells, 1993), have been shown to support pig oocyte maturation *in vitro*. Funahashi *et al.,* (1994b, 1996) found that cytoplasmic maturation of pig oocytes is significantly affected by the maturation medium after comparing TCM-199 with mWM and mWM with NCSU-23 medium during oocyte maturation. Also, Wang *et al* (1997) reported that significant differences were observed in glutathione content, cortical granule exocytosis, blastocyst development and number of cells in blastocysts in oocytes after *in vitro* fertilisation (IVF) when the oocytes were matured in NCSU-23 medium, TCM-199, or a modified Whitten's Medium. From this experiment, NCSU-23 medium seems to be the best medium for pig oocyte maturation. Perhaps, some basic media such as TCM-199 may not be suitable for pig oocyte maturation, or pig oocytes that are matured in different media may reach MII stage at different times.

In addition, several results clearly indicate that the addition as substrate of glutathione to the maturation medium is an important factor for male pronuclear formation of pig oocytes after sperm penetration. Yoshida *et al* (1992a) found that the rate of male pronuclear formation was significantly higher in oocytes matured in Waymouth MB 752/1 with or without 10% pig follicular fluid than in those matured in modified TCM 199 (MTCM-199) or Modified TLP-PVA. Waymouth medium contains a higher concentration of glutathione (GSH) and cysteine, precursor of GSH, than mTCM-199. It has been suggested that the synthesis of GSH during oocyte maturation is a prerequisite for male pronuclear formation in mouse (Calvin *et al* 1986). Furthermore, when a small volume of GSH solution (240 mmol/l) was microinjected into pig oocytes matured in mKRB solution, the rate of male pronuclear formation was higher (52-53%) than in non-injected oocytes (17-23 %) (Naito., *et al.* 1992). GSH is known to have an important role in maintaining the redox state of cells and in protecting them from harmful effects of oxidative injuries (Nagai, 1996).

### 1.2.2. Effect of hormones and growth factors

Addition of hormones to maturation medium is essential for successful oocyte maturation *in vitro* although some oocytes can reach the MII stage after being cultured in hormone-free media. Normally, pig oocytes are cultured in maturation media supplemented with ether hCG, FSH, eCG, LH, oestradiol or their combinations. Meinecke and Meinecke (1979) reported beneficial effects of gonadotrophins on nuclear maturation of pig oocytes and cumulus expansion. Yoshida *et al.,* (1992) also found that eCG and hCG alone or in combination, irrespective of the addition of

oestradiol, promoted nuclear maturation of pig oocytes compared with no hormones in maturation medium, however, there is an absolute requirement for eCG, or eCG+hCG for cumulus expansion or occur. In addition, Mattioli *et al.,* (1991) showed that FSH and LH separately or in combination induced cumulus expansion and accelerated the resumption of meiosis of pig oocytes. However, the percentage of oocytes with male pronuclei after maturation in the presence of LH and penetrated *in vitro* was nearly twice that observed in those matured in FSH or with no hormones. It seems to be that LH is more effective than FSH for both nuclear and cytoplasmic maturation of pig oocytes. Interestingly, Funahashi and Day (1993) found that the removal of hormone supplements in modified TCM-199 medium at 20 h after the start of culture enhanced the ability of male pronuclei 10-12 h after insemination. Their results showed that 67% of the penetrated oocytes formed male and female pronuclei, which was significantly higher than those obtained from maturation medium supplemented with hormones for an additional 20 h. One possible explanation may be that the intercellular communication is maintained by the metabolic stimulation of cumulus cells that was caused by the change of normal conditions during the second half of maturation. Because this is supported by the evidence that an active intercellular communication between cumulus cells and oocyte was seen for 30–40 h of culture although decreasing at 32 h after culture when oocyte-cumulus complexes were cultured in medium conditioned by follicle walls. This communication is similar to that of oocyte maturation *in vivo* (Motlik *et al.*, 1986).

In summary, LH seems to be more important than other hormones for oocyte maturation *in vitro* in pigs compared with the other species. Also, it is suggested that cytoplasmic maturation and cumulus expansion be greatly improved when the oocyte-cumulus complexes are cultured in a medium with hormonal supplements for 20 h followed by 20 h culture without hormones.

Epidermal growth factor (EGF) has been reported to stimulate nuclear maturation in pig oocytes (Grupen *et al.,* 1997). Ding, *et al* (1994) suggested that EGF might have a physiological role in the regulation of cytoplasmic maturation of pig oocytes. In addition, Illera *et al* (1998) found that the supplementation of EGF alone or in combination with insulin-like growth factor I (IGF I) in maturation medium, significantly increased the proportion of monospermic oocyte forming two normal pronuclei. Furthermore, Abeydeera, *et al* (1998) concluded that EGF at certain concentrations in maturation medium could influence the developmental competence of oocytes; he also proved that addition of EGF during the culture of pig embryos derived from oocytes matured in the presence of EGF had no effect. 18 piglets born from *in vitro* production support that embryos derived from oocytes matured in a medium containing EGF are viable (Abeydeera *et al.,* 1998). EGF indirectly influences maturation of pig oocytes through cumulus cells, since no effect is found in cumulus-free oocytes (Wang *et al.,* 1995).

### 1.2.3. Effect of serum and pig follicular fluid

Serum

Serum such as fetal calf serum (FCS), and newborn calf serum is commonly used for oocyte maturation. However, there is some evidence showing that FCS (5-100%) inhibits maturation of pig oocytes and cannot improve male pronuclear formation after sperm penetration *in vitro* when added to maturation medium supplemented with FSH (Naito *et al.*, 1988). Vatzias and Hagen (1999) also found that low polyspermy rates were observed for oocytes matured with 10% pig follicular fluid compared with those matured with 10% FCS although nuclear maturation was not significantly different between the oocytes matured with 10% pFF and 10% FCS. Based on these data, it seems to indicate that FCS in maturation medium could increase polyspermy after *in vitro* fertilisation (IVF).

Pig follicular fluid

Addition of pig follicular fluid (pFF) into maturation medium seems to be an important improvement in pig oocyte maturation. Vatzias and Hagen (1999) observed that pFF from small and large follicles inhibited maturation of pig oocytes *in vitro* when the maturation medium was not supplemented with gonadotropins and steroids, apparently the result of an adverse effect on nuclear and cytoplasmic maturation. In addition, Naito *et al* (1988) also found that only 36% of pig oocytes with cumulus cells matured to MII stage when they were cultured for 48 h in pFF obtained from medium-sized follicles, whereas 85-89% did so if FSH or hCG was added to the follicular fluid. After *in vitro* fertilisation, the proportion of fertilised oocytes with a male pronucleus also increased to 81% if the oocytes were matured in pFF and FSH. Yoshida *et al* (1990) proved that one or more heat-labile (56°C) acidic factors in pFF with a molecular

mass between 10 and 200 kDa were responsible for oocyte maturation. However, the lower polyspermy rate observed after addition of the highly purified fractions suggested that the same factor(s) played a role in reducing polyspermy. Furthermore, a report by Funahashi *et al* (1993) produced evidence of a high rate of male pronuclear formation in pig oocytes cultured for 20 h in medium supplemented with 10% pFF and gonadotropins (PMSG + hCG) and oestradiol, followed by 20 h culture in hormone-free medium with 10% pFF. This improvement may be due to a better synchronisation of nuclear and cytoplasmic maturation in pig oocytes during maturation *in vitro*. Taken together, it could be concluded that pFF has beneficial effects on pig oocyte maturation and developmental capacity *in vitro*. Vatzias and Hagen (1999) compared effects of different types of pig follicular fluid on pig oocyte maturation and development, and concluded that snap-frozen follicular fluid from medium follicles and conditioned medium from cultured oviducts of periovulatory gilts improve *in vitro* maturation, reduce polyspermy, and increase normal fertilisation rate *in vitro*.

Recent evidence has revealed that $Ca^{2+}$ release mechanisms are modified during oogenesis. The maximal sensitivity of $Ca^{2+}$ release is reached in the final stages of oocyte maturation, just before the optimal time for fertilisation (Carroll *et al.,* 1996). The process of oocyte maturation *in vivo* is stimulated by the periovulatory surge of gonadotrophins, while *in vitro* it occurs spontaneously when the fully-grown oocyte is removed from the inhibitory follicular environment to a suitable culture medium. Therefore, it is relevant that *in vitro* maturation system is not only an effect on nuclear

and cytoplasmic maturation, but also an effect on the development of $Ca^{2+}$ release mechanisms in oocytes.

## 1.3. Oocyte Activation at Fertilisation

In animals the interaction of sperm and egg membranes ultimately gives rise to a series of cellular responses in the egg that are required to initiate embryonic development. These responses occur in a temporal fashion and are classified as " early and late" events. Early events include the transient rise in intracellular $Ca^{2+}$ and the consequent cortical granule (CG) exocytosis. The contents of these CGs modify the extracellular coats surrounding eggs and result in a block to polyspermy. Later events include resumption of meiosis (and cell cycle), changes in intracellular pH, recruitment of maternal mRNAs, pronucleus formation, initiation of DNA synthesis, and cleavage (Schultz and Kopf, 1995). These events are collectively referred to as **oocyte activation**. The same or a similar sequence of events in fertilised oocytes can also be mimicked by activating unfertilised oocytes with artificial methods

### 1.3.1. Hypothesises of oocyte activation at fertilisation

Basically, regarding mammalian oocyte activation two hypotheses have been proposed to explain egg activation. The first is based on plasma membrane interaction (Miyazki *et al.,* 1993; Gilbert 1994; Foltz, 1995; Schultz and Kopf, 1995). When the sperm binds to receptors on the oocyte plasma membrane it is thought that this

stimulates a phospholipase C (PLC) that generates $InsP_3$, which then releases $Ca^{2+}$ (Miyazaki *et al.*, 1993). The sperm–receptors may stimulate phospholipase C via either tyrosine kinases or G-proteins. It is suggested that the spermatozoon may use a G–protein–coupled receptor, or a tyrosine (Tyr) kinase receptor. Although the receptor itself has not yet been identified, studies using specific activators or inhibitors support either of the two receptor–mediated models. It has been demonstrated that complete activation of mouse egg in the absence of spermatozoa can be achieved by stimulation of an exogenous heterotrimeric and monomeric G-protein-coupled receptor (Williams *et al.*, 1992; Moore *et al.*, 1993; 1994). Strong support for the G-protein model has been received from inhibition studies. Microinjection of the G-protein antagonist, GDPßS, into hamster or mouse eggs blocked several aspects of sperm-induced egg activation (Miyazaki, 1988; Moore *et al.*, 1994). Furthermore, some aspects of egg activation can occur through the Tyr kinase receptor pathway (starfish: Shilling *et al.*, 1994; Xenopus: Yim *et al.,* 1994; sea urchin: Abassi and Foltz, 1994), yet the Tyr kinase receptor in mammals has not been examined (Ben-Yosef *et al.,* 1998). Also, $InsP_3$ injection can cause $Ca^{2+}$ release in oocytes from a number of species (Miyazaki, 1988; Swann, 1992). The second is based on the introduction of a sperm factor into the egg cytoplasm (Parrington *et al.*, 1996). The sperm introduces a soluble factor into the oocyte after gamete membrane fusion and this factor triggers $Ca^{2+}$ changes (Swann, 1990; Stice and Robl, 1990; Stricker, 1996; Fissore *et al.,* 1998). Direct evidence for this hypothesis comes from the finding that injecting soluble sperm extracts made from boar, hamster, or human can trigger $Ca^{2+}$ oscillations in mouse, hamster, human and bovine oocytes

(Swann, 1990; 1992; 1994; Homa and Swann, 1994; Wu *et al.,* 1997; Palermo *et al.,* 1997; Fissore *et al.*, 1998). The sperm factor induced $Ca^{2+}$ oscillations are persistent, large amplitude oscillations that are similar in character to those seen during fertilisation (Swann, 1990; 1994). Injecting sperm extracts also causes other signs of activation such as cortical granule exocytosis, decreases in H 1kinase, and development up to the blastocyst stage (Stice *et al.*, 1990; Wu *et al.*, 1998; Fissore *et al.,* 1998). Using two independent fluorescence methods and cofocal microscopy, Lawrence *et al* (1997) demonstrated that sperm-egg fusion is the prelude to the initial $Ca^{2+}$ increase at fertilisation. They estimated the time interval between sperm-egg fusion and the onset of the $[Ca^{2+}]_i$ oscillations as 1-3 min. In addition, the clinical intracytoplasmic sperm injection procedure (ICSI) further supports the concept of the spermatozoon containing an activating molecule in its cytosol. The ICSI procedure results in full egg activation, as well as normal embryonic development, although no contact between spermatozoon and egg membranes occurs. At present a combination of both hypotheses appears more conceivable. It is also possible that the sperm receptor is composed of multiple molecules that activate more than one signaling pathway (reviewed by Whitaker and Swann, 1993; Schultz and Kopf, 1995; Kline, 1994). To date many signaling molecules including G-proteins, tyrosine kinases, the $IP_3$ receptor, and a variety of second message molecules have been found in eggs, but what sparks them and if and how they are networked for activation remain largely unknown (Snell and White, 1996).

A more recent study shows that injection of a functionally inhibitory antibody against Gq family G-proteins does not block activation at fertilisation in mouse eggs

(Williams *et al.*, 1998), which indicates that it is unlikely that these proteins are used by the sperm to initiate egg activation at fertilisation. Therefore the real functions of G proteins at fertilisation have to be revaluated. In addition, another recent discovery has provided evidence against the previous hypothesis that phospholipase C (PLC) activity introduced into the mouse egg as a consequence of sperm-egg fusion is responsible for causing $Ca^{2+}$ release. The discovery demonstrated that the minimum PLC activity, from purified PLC γ1 and protein, needed to elicit $Ca^{2+}$ release when injected into eggs was approximately 500-900 times the PLC activity contained in a single sperm (Mehlmann *et al,* 2001). Therefore, this indicates that a single sperm does not contain enough PLC γ1 activity to be responsible for causing $Ca^{2+}$ release at fertilisation. Similarly, It has been also confirmed that $Ca^{2+}$ release at fertilisation of mouse eggs does not require SH2-domain-mediated activation of PLC γ, whereas the injected SH2 domain constructs were thought to compete with the SH2 domains of PLCγ in the egg, blocking its activation by tyrosine kinase (Mehlmann et al., 1998). Taken these results together, at least the pathways of G-proteins and PLC γ involving fertilisation in mouse, which were thought to be important for oocyte activation at fertilisation, have been challenged. The mechanisms by which the sperm triggers $Ca^{2+}$ release from the mature oocyte at fertilisation seem to be more complicated than we thought.

### 1.3.2. Patterns o f $Ca^{2+}$ oscillations

All eggs are activated at fertilisation by an increase in intracellular free $Ca^{2+}$. It is suggested that the release of free $Ca^{2+}$ play a very important role in activation of the

eggs. Dynamic measurements of $[Ca^{2+}]_i$ revealed a marked change accompanying fertilisation of the mammalian eggs. This first fertilisation $Ca^{2+}$ transient takes the form of a wave originating at the point of sperm entry (Miyazaki, 1988). In mammals, the first distinctive transient is followed by a series of $Ca^{2+}$ oscillations of high amplitude and short duration that persist for several hours. The basal $[Ca^{2+}]_I$ in ovulated MII arrested mammalian eggs is approximately 100 nmol $l^{-1}$, a characteristic value within many different inactive somatic cells. The initial $Ca^{2+}$ transient is variable in its amplitude and duration, although it is usually lower and lasts longer than the subsequent transients (duration 3-6 min, with amplitude of approximately 3-4 times the basal level). This first transient is followed by a series of narrower $Ca^{2+}$ transients (duration 0.5-1.5 min) of high amplitude (up to 6-8 times the basal level). Rodent eggs, as well as those of humans, exhibit high frequency oscillations (regular peak to peak intervals ranging between 2 and 4 min), whereas in bovine and porcine eggs 15-30 min elapse between two peaks (Ben-Yosef *et al.*, 1996). However, more recent data show that the peak-to-peak intervals of $Ca^{2+}$ transients in mouse eggs at fertilisation last approximately 20 min rather than 2-4 min reported before, which is similar to those in pig and bovine oocytes (Swann and Parrington, 1999). The phenomenon of cyclical $Ca^{2+}$ transients in *in-vivo* fertilised eggs has been shown to reflect the physiological events that occur during fertilisation in mammals (Ben-Yosef., *et al.*, 1993). Also, these patterns of $Ca^{2+}$ oscillations in mammalian oocytes at fertilisation may be important for evaluating and improving artificial methods of oocyte activation

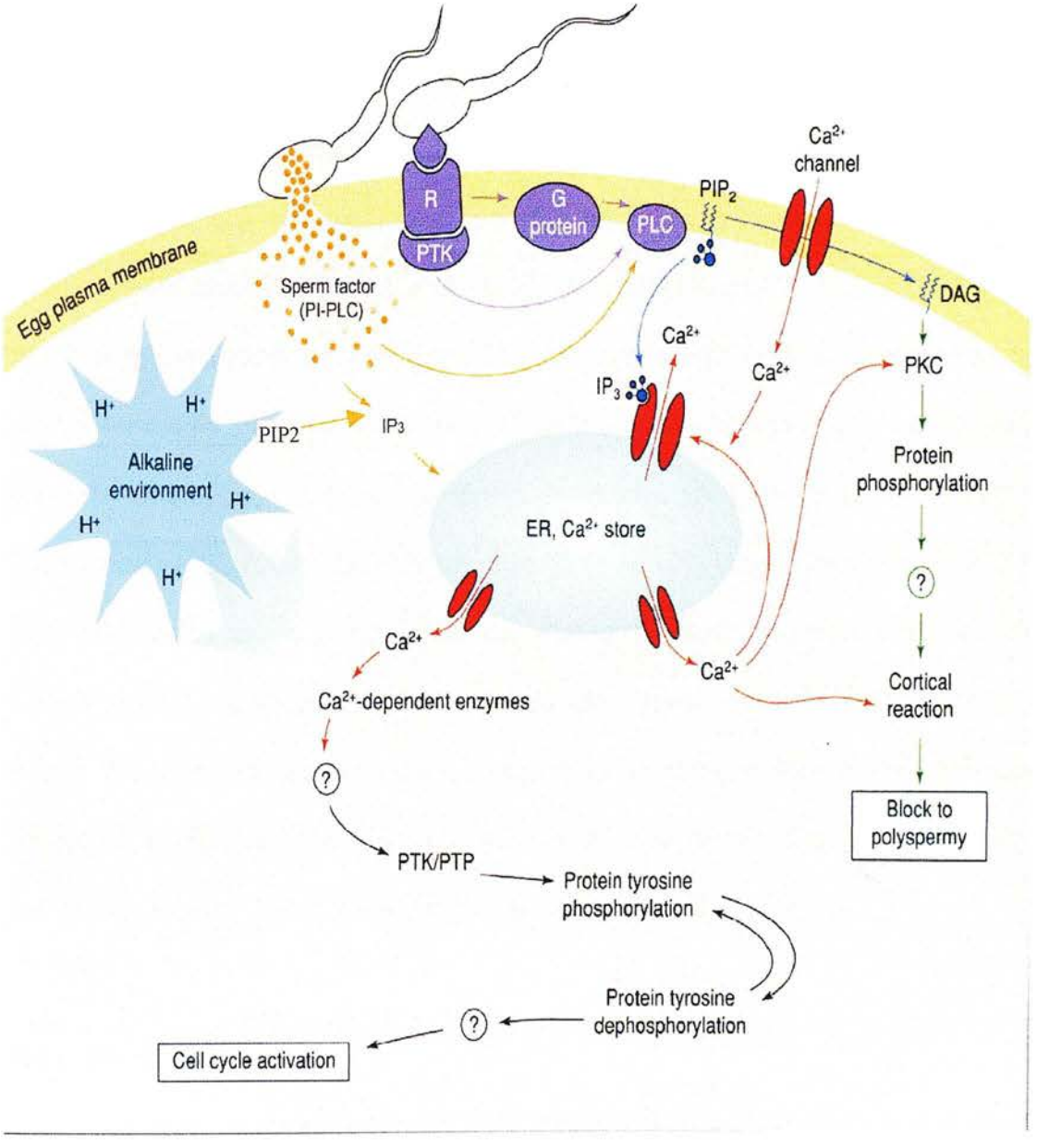
A single $Ca^{2+}$ transient is sufficient to cause exocytosis of most of the cortical granules, leading to an effective block to polyspermy as well as inducing resumption of the second meiotic division (Jaffe, 1985; Tombes *et al.*, 1992; Vincent *et al.*, 1992; Kline and Kline, 1994). Moreover, blocking the increase of fertilisation-induced $[Ca^{2+}]_i$ inhibits egg activation (Kline and Kline, 1992). However, microinjecting mouse eggs with physiological concentrations of inositol 1,4,5-tris-phosphate ($IP_3$), resulted in cortical granule exocytosis (CGE) but not in emission of the second polar body or pronuclear formation (Kurasawa *et al.*, 1989). Fine-tuning of the single $Ca^{2+}$ transient demonstrates that low $Ca^{2+}$ concentrations allow CGE, while higher concentrations are required for cell cycle resumption (Raz *et al.*, 1998). Therefore, it is conceivable that cell cycle progression is dependent upon the temporal and spatial aspects of the $Ca^{2+}$ transient. Most activators of $Ca^{2+}$ release induce a larger $Ca^{2+}$ increase than the initial response at fertilisation, thus leading to complete activation of the egg. It is possible that the first physiological $Ca^{2+}$ transient is not adequate for complete egg activation, and additional transients are required. In two studies, Ozil (1990) stimulated the normal sperm-induced $Ca^{2+}$ transient by repeated electric field pulses, evoking $Ca^{2+}$ increases at a frequency similar to that seen at fertilisation (Ozil, 1990; Vitullo, 1992). They suggested that the oscillations might somehow be coupled to events occurring during the first cell cycle, and come into play when a prolonged signal is required after a single stimulus, as in fertilisation. However, most artificial activators cause only a single, lager $Ca^{2+}$ transient in the egg. It is seemed that the patterns of $Ca^{2+}$ transients may not only affect egg activation but also developmental competence of the activated egg.

It is believed that the site of $Ca^{2+}$ release and sequestration is the endoplasmic reticulum. Mehlmann *et al* (1995) demonstrated that competence to undergo normal activation at fertilisation is associated with a marked reorganisation of the egg endoplasmic reticulum, and the development of the $IP_3$-induced $Ca^{2+}$ release system. Nevertheless, two types of mechanism can mediate $Ca^{2+}$ release from intracellular stores: $IP_3$ induced $Ca^{2+}$ release (IICR) mediated by the $IP_3$ receptor, and $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) mediated either by the ryanodine or by the $IP_3$ receptor, in other words, The $Ca^{2+}$ stores are gated by two families of $Ca^{2+}$ channels, the inositol 1,4,5-trisphosphate ($InsP_3$) receptor and the ryanodine receptor (RyR) (Lai *et al.,* 1988; Furuichi *et al.*, 1989). A suggested mechanism of sperm–induced egg activation has been summarised by Ben-Yosef and Shalgi (1997) (Fig 1.3.). It could be imagined that a change of any element in controlling the pathways, which could cause the release of $Ca^{2+}$ from cellular stores, would potentially result in activation of the egg. Therefore, most of the currently used activation procedures rely on different methods to induce an intracellular $Ca^{2+}$ release. These procedures include (1) an electrical pulse (Tarkowski, 1975; Ware *et al.*, 1989; Ozil, 1990). (2) Ethanol (Cuthbertson *et al.,* 1981; Nagai, 1987); and (3) $Ca^{2+}$ Ionophore and Ionomycin (Steinhardt *et al.*, 1974; Ware *et al.*, 1989; Kline and Kline, 1992; Loi *et al.*, 1998).

**Fig.1.1. Modified suggested mechanism of sperm-induced egg activation**

Binding of a spermatozoon to a G-protein-coupled receptor (G-protein) or, alternatively, to a tyrosine-phosphorylated (PTK) receptor (R) induces an increase in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]i$). A soluble sperm protein (oscillin) can also be responsible for this $Ca^{2+}$ response. The source of $Ca^{2+}$ for the transients originates from the endoplasmic reticulum (ER). Moreover, the continuous oscillations also require an influx of $Ca^{2+}$ from outside to refill the previously emptied stores. The $[Ca^{2+}]i$ increase induces resumption of meiosis and activation of the cell cycle in one direction, and cortical granule exocytosis leading to the block to polyspermy, in another direction. The increase in $[Ca^{2+}]i$ activates $Ca^{2+}$-dependent enzymes, which can facilitate activation of non-receptor protein tyrosine kinases (PTK) or inactivation of protein tyrosine phosphatases (PTPs). Phosphorylation of specific substrate proteins further transduces the sperm signal to downstream cell cycle regulators. The naturally high $pH_i$ within the ovulated egg provides a suitable environment in which the enzymes activated by the $Ca^{2+}$ transient can function. Cortical reaction can be triggered by the initial $Ca^{2+}$ increase, or by a $Ca^{2+}$-dependent protein kinase C (PKC). DAG, diacylglycerol; $IP_3$, inositol 1,4,5-tris-phosphate; $PIP_2$, phosphatidylinositol 4,5-biphosphate; PLC, phospholipase C. PI-PLC, phosphoinositide specific PLC.

This diagram is cited from D.Ben-Yosef and R. Shalgi (1998) with some modifications.


Egg plasma membrane
Sperm factor
(PI-PLC)
R
PTK
G
protein
PLC
PIP2
Ca2+
channel
DAG
PKC
Protein
phosphorylation
?
Cortical
reaction
Block to
polyspermy
Ca2+
IP3
PIP2
IP3
H+
Alkaline
environment
ER, Ca2+ store
Ca2+
Ca2+-dependent enzymes
?
PTK/PTP
Protein tyrosine
phosphorylation
Protein tyrosine
dephosphorylation
?
Cell cycle activation

## 1. 4. Artificial Methods of Oocyte Activation

In addition, new agents are continuously discovered or created. However, because some treatments or agents such as cool or heat damage oocytes, they are inefficient or not practical, so only a few treatments and agents (see those underlined in Table 1.3.) can commonly be used for oocyte activation. On one hand, some treatments or agents seem to be species-specific, for instance, $Sr^{2+}$ can effectively activate mouse oocytes but not pig, cattle and sheep oocytes; on the other hand, some agents such as sperm protein factor(s), which seem to be non-species-specific, can activate oocytes from all species. Most successful results on oocyte activation from publications have been achieved by electrical stimulation, as well as chemicals such as $Sr^{2+}$ in mouse and ethanol, Ionomycin, calcium ionosphere in mouse (Kishikawa et al, 1999), pig (Betthauser *et al.*, 2000), cattle (Susko Parrish *et al* 1994) and sheep (Loi *et al* 1998). To date we have known many factors influencing the result of oocyte activation could include quality and age of oocytes, used method or agent, type of activation media and oocyte pre-treatment, concentration of agents or strength of stimulation, duration of treatment or stimulation, activation conditions such as temperature, pH, osmolarity in activation medium. These factors will be discussed later in this review.

### 1.4.1. Electrical activation

Application of strong electric field pulses to cells is known to cause some type of structural rearrangement of the cell membrane. Significant progress has been made

by adopting the hypothesis that some of these rearrangements consist of temporary aqueous pathways ("pores"), with the electric field playing the dual role of causing pore formation and providing a local driving force for ionic and molecular transport through the pores. Cell stress probably occurs because of relatively non-specific chemical exchange with the extracellular environment. Whether or not the cell survives probably depends on the cell type, the extracellular medium composition, and the ratio of intra- to extracellular volume (Weaver, 1995). $Ca^{2+}$ is thought to be a key mediator of oocyte activation. Therefore, it is relevant that electrical pulses induce oocyte activation via, presumably, $Ca^{2+}$ uptake through membrane pores. In addition, evidence using erythrocyte membrane indicates that two classes of pores must be considered at electric pulsing (Sowers and Lieber, 1986). Large pores, reaching a radius of at least 8.4 nm, open, but immediately reclose within 100 – 200 msec. A second class of pores, with a radius of about 0.5 nm, stays open for an indefinite period of time. These small pores may not be due to incomplete closing of the large pores (Robl *et al.*, 1992). In human red blood cells, it has been found that a few seconds after the electric pulse, the electric field – induced pores appeared to have begun to reseal and that diameters also appeared to be smaller. At about half a minute after the electric pulsing, the membrane in most red cells appeared almost like that of control red cells (Chang, 1992). In another experiment on the time course of formation of electricopores with rabbit oocytes, the data showed that uptake of NBD-G, a low molecular weight dye (347 Da, radius of 0.45 nm) was detected up to 40 min after a single pulse of 3.6 kV/cm for 60 μsec and uptake tended to be higher than in control nonpulsed oocytes from 15-25 min (Collas et al

1993). These data suggests that membrane recovery is often orders of magnitude slower, and that pores created by electrical pulses would take second – minutes or even hours to reseal after electric pulses.

Electrical stimulation has been used to parthenogenetically activate oocytes in all animals, especially for activating and fusing nuclear-transferred embryos. Electrical stimulation, although not necessarily more effective than other treatments, is convenient

**Table1.3. List of physical and chemical stimuli inducing oocyte activation in mammals**

| PHYSICAL | CHEMICAL | BIOLOGICAL |
|---|---|---|
| 1.Mechanical<br>a. Pricking<br>b. Manipulation of oocyte in vitro.<br><br>2. Thermal<br>a. Cooling<br>b. Heating<br><br>3. Electric | 1.Enzymatic:<br>tyrosine, pronase, hyaluronidase<br>2. Osmotic<br>3. Ionic: divalent cations, calcium ionophore<br>4. Anaesthetics<br>a. general:<br>ether, ethanol , nembutal, chloroform, avertin.<br>b. local:<br>dibucaine, tetracaine, lignocaine, lidocaine chlorpromazine.<br>5. Phenothiazine, tranquillizers, thiordazine, trifluoperazine, Fluphenazine, chlorpromazine.<br>6. Protein synthesis inhibitors:<br>cycloheximide, puromycin | Sperm protein factor<br>G-proteins<br>$InsP_3$(Inositol,1,4,5-trisphosphate) |

Note: the underlined treatments and agents are commonly used at the present

and easier to manipulate. Because an electrical pulse can be used both to fuse and to activate nuclear transferred embryos; electrical fusion and activation are essentially the only method being used in nuclear transfer. Thus, most of cloned farm animals were produced using electrical stimulation, including Dolly. Electroporation is a phenomenon in which the membrane of a cell exposed to high-intensity electric field pulses can be temporarily destabilised in specific regions of the cell. During the destabilisation period, the cell membrane is highly permeable to exogenous molecules present in the surrounding media. Electroporation thus can be regarded as a massive microinjection technique that can be used to inject a single cell or millions of cells with specific components in the culture medium (Chang *et al.,* 1992). It was found that if the electrical field was applied as a very short duration pulse, the cells could recover from the electrical treatment. This implied that these electric field-mediated "pores" were resealable and could be induced without permanent damage to the cells (Baker and Knight, 1978a; 1978b; Gauger *et al.,* 1979; Zimmermann *et al.,* 1980).

Electrical stimulation is thought to cause oocyte activation by inducing an elevation of intracellular calcium. The source of the $Ca^{2+}$ rise is exclusively extracellular, and a single pulse generates a single $Ca^{2+}$ rise (Fiossore and Robl, 1992) The amplitude of the generated $Ca^{2+}$ rise is dependent on the concentration of $Ca^{2+}$ in the extracellular media, and on the duration and amplitude of the pulse (Fiossore and Robl, 1992; Ozil and Swann, 1995). Calcium seems to be an essential component of cell membranes and its displacement may produce sufficient modification of the vitelline

membrane to trigger oocyte activation. The vitelline membrane in mouse oocytes becomes much more resilient and difficult to penetrate with microelectrodes when $Ca^{2+}$ ions are omitted from the external medium (Whittingham, 1980). Although the source of $Ca^{2+}$ during electrical activation is generally believed to be extracellular, it was suggested that electroporation also stimulates $Ca^{2+}$ release from intracellular stores (Machaty *et al.,* 1999) because recently reported results (Wang *et al.,* 1999) show that oocytes can be activated in $Ca^{2+}$ free medium. However, in some cases activated oocytes show a response that is similar to the response of oocytes that do not activate. Also, the level of calcium response does not relate to activation. The release of $Ca^{2+}$ in an oocyte is not a reliable indicator to assess whether the oocyte is activated or not (Fiossore and Robl, 1992).

#### 1.4.1.1. Effect of age of oocytes

Most of the current activation procedures including electrical activation are able to activate mammalian oocytes, but the rates of activation are highly dependent on the age of the oocytes. Recently ovulated or young oocytes are not effectively activated. It has been confirmed that activation rate increases with ageing of the oocyte in several species, such as cow, mouse and rabbit. Leal *et al* (1998) electrically activated pig oocytes matured *in vitro* for either 36 or 48 h, and found that pronuclear formation was significantly lower in newly matured (36 h) than aged oocytes (34% vs. 85%). They also suggested that the ability of pig oocytes to become fully activated might be related to the modification of H1 kinase activity during the ageing process. In addition, Hagen

*et al* (1991) activated pig oocytes at 24, 31, 41, 48, and 65 h of incubation; the activation rates were 0, 0, 87, 88, and 83 %, respectively. Likewise, similar work has been reported by Yamauchi *et al* (1996) who matured pig oocytes for 24, 30, 36, 42, and 48 h in TCM199 with Earle's s salt supplemented with 10% fetal calf serum before electrical stimulation. The results showed that the proportions of activated oocytes were 5.4, 6.1, and 93.2% at 24, 30, and 42 h of maturation, however, when the culture period was extended to 48 hours, there was a significant decrease to 56.7%. Kikuchi *et al* (1995) activated pig oocytes cultured at 30, 60 and 70 h of maturation, respectively, the activation rates increased with ageing of oocytes (7, 46 and 57%). Also, Kim *et al* (1997) found that the percentage of activated oocytes with one female pronucleus was higher in oocytes at 40 h of maturation than that at 50 or 60 h of culture. In contrast to these data, Jolliff and Prather (1997) reported that pig oocytes matured in Waymouth's medium and electrically stimulated at 36 h developed to the same degree as oocytes stimulated at 48 h. Although there were some differences including maturation and activation methods among these experiments, most observations showed that activation rate of pig oocytes activated by electrical stimulation is also age-dependent. In addition, at least in the bovine, increased activation rates with oocyte age cannot be due to decreased levels of maturation promoting factor (MPF) (Collas *et al.,* 1993). Recently, Carroll *et al* (1996) suggested that the tight–co-ordination of the differentiation of the $Ca^{2+}$ signalling system with the development of the oocyte provides a means of ensuring successful activation at the time of fertilisation. They also pointed out that a clear demonstration that the $Ca^{2+}$ signalling system was developmentally regulated was that

the ability of spermatozoa to cause $Ca^{2+}$ transients depended on the stage of maturation of the oocyte. It has been found (Carroll *et al.,* 1996) that in mature oocytes, $Ca^{2+}$ transients were larger, had a faster rate of rise and continued for longer than in immature oocytes. A possible explanation to the age-dependent effect of oocytes is that the inherent properties of the $Ca^{2+}$ channels, the size of the $Ca^{2+}$ store and the density of the $Ca^{2+}$ channels are all expected to increase the sensitivity of regenerative $Ca^{2+}$ release during maturation (Carroll *et al.,* 1996) and decrease the occurrence of spontaneous $Ca^{2+}$ oscillations (Carroll and Swann, 1992; Fujiwara *et al.,* 1993). Nevertheless, the age-dependent effect of oocytes may, at least in part, reflect the development and differentiation of $Ca^{2+}$ signalling system in the oocyte during maturation, especially at metaphase stage. It is clear that the maturation system can be an important effect on the "maturation" of the $Ca^{2+}$ signalling system. From this point, the best way to improve activation of oocytes would be to improve maturation and synchronisation of oocytes.

#### 1.4.1.2. Effect of electrical field strength

The electric generator provides generally two electric signals: (1) continuous alternating high-frequency electric field (AC) to align cells into pearl chains, and (2) short, unipolar pulses ( often called DC pulses, singnals which show only one polarity) of high amplitude. For electroporation, only the short, unipolarity high electric field pulses are required. The alignment effect is stronger with a square wave of the same amplitude as a sine wave, because the cells are exposed to the maximum amplitude for a longer period time. Square-wave direct current (DC) pulse(s) of kV/cm is commonly

used with pulse duration of μs or ms for either cell fusion or oocyte activation (Hofmann, 1989). The electrical pulse(s) can create pore formation on plasma membranes of oocytes, through which $Ca^{2+}$ enters oocytes from the activation medium and triggers the release of $Ca^{2+}$ from intracellular stores, resulting in activating the mature oocyte.

Prochazka *et al* (1992) investigated effect of a single pulse of different electrical field strengths on pig oocyte activation, and found that a single direct current (DC) pulse of 0.75-1.65 kV/cm for 30 or 100 μsec activated >90% of matured oocytes, the 2 lowest effective stimuli (0.45 and 0.60 kV/cm for 30 μsec) frequently produced oocytes that remained in pronuclear stages of activation (29.4 and 42.3%, respectively), whereas the strongest stimulation (1.05-1.65 kV/cm for 100 μsec often yielded oocytes that failed to extrude the 2nd polar body and formed ≥2 pronuclei ( up to 56.3%). They also suggested that the optimal single pulse of electrical field strength to activate pig oocytes was 0.75 kV/cm. Collas *et al* (1993) proved that the level of electrical stimulation influenced oocytes $Ca^{2+}$ response, activation, and parthenogenetic development in cows. For example, in the cow, a single pulse of 0.2 or 1.0 kV/cm DC did not affect activation frequency (65 vs. 68%, $p>0.1$) and had a limited effect on rate of parthenogenetic development to blastocysts (5 vs. 12%, $P<0.05$) (Collas *et al.,* 1992). In fact, according to scientific articles published (Jolliff and Prather, 1997; Leal *et al.,* 1998; Wang *et al.,* 1999; Hagen *et al.,* 1991), the electrical field strengths used for pig oocyte activation normally range from 1.0 to 1.5 kV/cm. However, electrical field strength is not a single effect, because it is always related to duration of pulse and pulse number. Perhaps, to

use different electrical field strengths combined with different duration of pulse and the number of pulses to activate oocytes, more effective combinations of electrical field strength and duration of pulse and the number of pulses could be found.

### 1.4.1.3. Effect of pulse number.

The rate of activation of oocytes is greatly improved with the use of multiple pulses. Activation rate was much more influenced by age of the oocyte and number of pulses than pulse parameter. This may be explained by the observation that all oocytes respond to an electrical pulse with a transient increase in intracellular calcium. Multiple electrical pulses presumably cause repeated calcium transients, which induce activation in oocytes not capable of responding to a single pulse (Robl *et al.,* 1992). For example, in the cow, three stimuli, as opposed to one, enhanced activation rate (88 vs. 65%, respectively, $P<0.01$) and parthenogenetic development to the blastocyst stage (5 vs. 22%, $P<0.001$). These results show that multiple pulses elicit multiple intracellular $Ca^{2+}$ elevations. These $Ca^{2+}$ transients may induce activation of oocytes not able to respond to a single stimulation, and may be beneficial for parthenogenetic development (Collas *et al.,* 1992). Also, Ozil and Swann (1995) suggested that activation by electrical stimulation could be obtained by repeated electric pulses that, if precisely controlled, may induce a pattern of calcium release similar to that induced by sperm penetration. In other words, multiple pulses result in multiple $Ca^{2+}$ elevations that are more important for resumption of MII oocytes. Grupen *et al* (1999) activated pig oocytes with two DC pulses (1.5 kV/cm, 60 μs) 1 sec apart in 0.3 M Mannitol solution containing 0.1 m*M*

$CaCl_2$, 0.1 m*M* $MgSO_4$ and 0.01% PVP, and gave additional sets of pulses 30 and 60 min after the first set. The results showed that the rates of blastocysts cultured for 7 days were 35, 49, and 23% for 1, 2 and 3 sets of pulses using ovulated oocytes, respectively. However, Kim *et al* (1997) activated pig oocytes with a single DC pulse of 1.2 kV/cm for 30 μsec, 24% of which developed to morulae/blastocysts. Similar results reported by Màchaty and Prather (1998) who activated pig oocytes matured for 42 h and 48 h by a 1.0 kV/cm DC pulse for 30 μsec, showed that the rates of morulae/blastocysts were 33.9 and 23.8% respectively, for young and aged oocytes after 6 days of culture. Furthermore, Hagen *et al* (1991) activated pig oocytes matured for 41 h by 0, 1, or 2 DC electrical pulses of 1.2 kV/cm for 30 μsec, respectively, confirming that 2 pulses halved the percentage of activated oocytes ($p<0.001$). In spite of the incomparable results, multiple pulses should show a tendency to be more effective than a single pulse, at least in theory. This has been proved in rabbits (Ozil, 1990).

#### 1.4.1.4. Effect of duration of stimulation

Compared with effects of electrical field strength and number of pulses, duration of stimulation seems to be not important in oocyte activation. Therefore, most of laboratories in the world use duration of electrical stimulation ranged from 30 to 80 μsec of DC pulse(s). Collas *et al* (1993) proved that with one or three pulses, increasing pulse duration from 20–100 μsec did not affect cleavage rate 28-48%, $P>0.05$) or parthenogenetic development to the blastocyst stage (9-14%, $P>0.1$). Using higher field strengths for a short duration may achieve the same activation rate as using low

electrical field strengths for a relatively long duration if same pulse(s) is employed. It may reduce damage to oocytes as well.

### 1.4.1.5. Effect of activation media

In addition, activation media in which the cells are suspended is subjected to the electric field generated by the voltage across the chamber electrodes. They normally have a low electrical conductivity in order to avoid damaging cells due to an increase of temperature (heat shock) or to excessive strong electrical energy produced in media with a high electrical conductivity at electrical activation. The specific resistivity of the medium is determined by the concentration of charge carriers (ions). Sugar (e.g., Mannitol or sucrose) solutions have a low charge carrier concentration and exhibit a high specific resistivity. They are also relatively compatible with cells at least for a limited amount of time if the sugar solution is isosmotic or hyperosmotic with respect to the cytosol of the cell. They are, therefore, a preferred medium for electrofusion and electroporation in cases where heating effects need to be minimised. Also, an electrolyte such as PBS exhibits non-linear electrical characteristics. Its specific electrical resistivity decreases with temperature and with increasing applied field (Hofmann 1989). Clearly, the medium and components of the medium must meet the requirements mentioned above and not be toxic to the cells. In addition, cellular stress caused by electroporation may lead to cell death without irreversible electroporation itself having occurred. Both reversible and irreversible electroporation result in transient opening (pores) of the membrane. These pores are often large enough that molecular transport is

expected to be relatively non-specific. In the case of reversible electroporation, significant molecular transport between the intra- and extra-cellular volumes may lead to a significant chemical or osmotic imbalance. If this imbalance is too large, recovery may not occur, with cell death being the result. In addition, cell death could also be led by rupture of an isolated portion of a cell membrane, which causes cell lysis (Weaver, 1994). Actually, the effect is mainly contributed by $Ca^{2+}$ concentration in activation medium other than activation medium. However, little is known of the specific requirements the medium for activation. Because the $Ca^{2+}$ transient caused by electrical stimulation is the result of an influx of calcium it would then be expected that calcium would be required in the medium. Ozil (1990) verified this in an experiment with rabbit oocytes in which none of 105 oocytes activated in medium without electrolytes whereas up to 100% activated with the addition of 10 $\mu M$ $CaCl_2$. The concentration of calcium in the medium determines the peak elevation in intracellular calcium. Furthermore, no spike of calcium in pig oocytes in activation medium without calcium was observed after electrical stimulation (Sun *et al.*, 1992). However, the calcium peak is not related to activation, the concentration of calcium in the medium may not be critical for activation (Robl *et al* 1992). In contrast to the results mentioned above, there is evidence that in mouse and pig oocytes a marked increase of $Ca^{2+}$ occurs even in the absence of extracellular $Ca^{2+}$, indicating that part of the $Ca^{2+}$ originates from intracellular stores (Shiina *et al.,* 1993; Wang *et al.,* 1999). $Ca^{2+}$ concentration in the medium is considered to be critical for oocyte activation. It has been verified that $Ca^{2+}$ concentration in activation medium can significantly influence oocyte activation and

even subsequent development. (Collas *et al.*, 1993), but too much $Ca^{2+}$ inside cells can be harmful, which indicates that an adequate $Ca^{2+}$ concentration in activation medium is very important for the success of oocyte activation. In addition, different activation media could have a different conductivity that could lead to different field strength of direct current activating oocytes when the same field strength are given. Taken all the data together, it is still question of whether free calcium in activation medium is essential for oocyte activation or not. However, $Ca^{2+}$ in activation medium has been confirmed to be beneficial for either activation rate or parthenogenetic development to the blastocyst stage (Wang *et al.*, 1999). At present, Mannitol or Sorbitol of 0.28–0.3 M, $Ca^{2+}$ of 0.05–0.1 mM and $Mg^{2+}$ of 0.1mM are normally added in activation media. Except these chemicals, small amount of BSA or Hepes is commonly supplemented in activation media in order to balance pH value or to prevent cell sick. However, oocytes from different species may require different activation conditions including concentration of these components. The answer seems to be certain because oocytes from different species were activated under the same conditions, but the results were completely different.

In addition, in comparing a chamber with 1 mm wire electrodes to a chamber with 1-mm rectangular electrodes, the chamber with rectangular electrodes seemed to be better than the former (Collas *et al.*, 1989).

### 1.4.2. Sperm protein factor

Recently a report (Fissore *et al.,* 1999) demonstrated that the relationship between improper oocyte activation and abnormal spindle function could be observed in parthenogenetically activated oocytes. They also pointed out that oocytes activated by electrical pulses, ionomycin, ionomycin plus DMAP, ethanol and likely other methods, did not induce a physiological pattern of calcium oscillations and did not consistently induce extrusion of a second polar body. The consequence of these activation methods usually causes damage of microtubules in the activated oocytes, resulting in abnormalities in the regulation of spindle function. Although electrical stimulation is practically and commonly used to activate oocytes in all species, actually it does not work well at all time because of the age-dependence of activation mentioned above. It activates aged oocytes more efficiently than young oocytes. However, Tanaka and Kanagawa (1997) reported that the use of recently ovulated or young oocytes as recipient cells could improve development of nuclear transfer embryos. In addition, electrical stimulation is clearly non-physiological. Thus, electrical field strength and pulse(s) must be tested in order to find adequate stimuli. But the tested stimuli are not always effective because of the use of oocytes at different ages. Alternatively, using sperm protein factor could overcome these problems.

Recently, a sperm protein factor has been extracted from sperm in several species, such as rabbits, rats, mice, pigs and human (Parrington *et al.,* 1996; Stice and Robl, 1990; Swann, 1994; Wu *et al.,* 1998). Once the protein is injected into unfertilised oocytes, $Ca^{2+}$ oscillations take place and are the same as those seen at fertilisation. Likewise, it can also induce $Ca^{2+}$ oscillations in somatic cells, such as neurones and

hepatocytes (Berrie *et al.,* 1996). The sperm protein factor is physiological compared to the other agents although the injected volume of the factor must be controlled precisely.

The sperm protein factor extracted from hamster was reported to exist as an oligomer with a subunit of *Mr.* 33 K and a specific intracellular localisation at the equatorial segment of the sperm head, also it was suggested that 33 K protein appeared not to be one of G-proteins by a panel of antibodies specific for the mammalian G-proteins Giα1, Giα2, Giα3, Goα, Gsα and G-protein β-subunits and that it had a soluble nature with an isoelectric point (pI) of ~6 (Parrington *et al.,* 1996). There are some arguments about its molecular weight. However, more recent work has shown that recombinant 33 kDa protein does not trigger $Ca^{2+}$ oscillations when injected into eggs (Shevchneko *et al.*, 1998; Woloskser *et al.*, 1998) and that presence of the 33 kDa protein does not always correlate with $Ca^{2+}$ oscillation inducing activity (Wu *et al.*, 1998). These data suggest that the 33 kDa protein is not a component of the oscillogen or sperm factor (Swann and Parrington, 1999). Also, molecules as large as 240 kDa have been found to diffuse into the egg from the sperm before $Ca^{2+}$ release occurs (Jones et al., 1998), which have been suggested to be a soluble cytosolic sperm factor that can cause the same $Ca^{2+}$ oscillations and activation after microinjection into oocytes as those seen at fertilisation. Meanwhile a mechanism of this sperm factor activating oocytes at fertilisation has been suggested that the sperm factor has a phosphoinositide specific phospholipoase C (PI-PLC) activity that leads to the generation of $InsP_3$ in eggs, subsequently triggering the entire series of $Ca^{2+}$ oscillations

through the $InsP_3$ receptor on the $Ca^{2+}$ stores after gamete-membrane fusion (Swann and Parrington, 1999).

The sperm protein factor has two significant advantages for activation of reconstructed embryos. The first advantage is that, unlike most artificial activation methods or agents that are only effective with aged oocytes, the sperm protein factor is able to activate recently ovulated oocytes that would benefit development of the nuclear transferred embryos (Stice and Robl, 1990). The second is that the sperm protein factor is physiological compared to the others such as ethanol, $Ca^{2+}$ Ionophore and strontium. Therefore, the sperm protein factor may cause a minimum damage of the activated oocytes. In addition, the sperm protein factor seems not to be species-specific, which means the factor extracted from one species can activate oocytes in all species following injected into cytoplasm of the oocytes. Thus, use of the sperm protein factor would, perhaps, provide not only more effective activation, but also improvement of subsequently embryonic development. In addition, some difficulties should be considered when the sperm protein factor is injected into MII oocytes. The first is that the sperm protein factor has to be injected into cytoplasm of individual oocytes, which takes much more time than the other methods; also, it requires an expensive manipulator and a skilful person. The second is that the exact amount of the sperm protein factor injected into every oocyte is difficult to control.

Obviously, these problems would limit the application of the sperm protein factor in animal cloning. Recently the sperm protein factor has been applied in cattle nuclear transfer (Knott *et al.*, 2001). After activation, 13% of nuclear-transferred

embryos (38/292) developed to the blastocyst stage, the blastocysts were transferred into 15 recipient animals. The pregnancy rates of 40 and 13% on day 30 and day 60 were obtained compared with 56 and 38% in the control in which nuclear transferred embryos were activated with 5 μM Ionomycin followed by 10 μg/ml cycloheximide for 5 h. Although either blastocyst rate or pregnancy rate was lower than those in the control, these data demonstrated for the first time that the sperm protein factor could be used for activating nuclear transferred embryos in animal cloning. In addition, especially since the first piglets were cloned from adult somatic cells with a serial nuclear transfer mentioned above (Polejaeva *et al.,* 2000), the serial nuclear transfer has been considered to have a benefit to the development of nuclear transferred embryos. It is likely that the current methods used to activate eggs following nuclear transfer are not optimal. Recent reports (Kono *et al.,* 1995; Polejaeva *et al.,* 2000; Ono *et al.,* 2001) have implied that co-injecting sperm extracts or purified molecules–once they are known–together with donor nucleus or injecting sperm protein extracts to activate nuclear transferred embryos might alleviate this problem. That means, the sperm protein factor might be a potential and ideal candidate to simplify the complex procedure of the serial nuclear transfer. It could be predicted that research into the sperm protein factor would intensively be carried out in the next years due to the sperm protein factor being only an ideal and physiological agent for oocyte or nuclear transferred embryo activation at the moment. Perhaps, after several years, sperm protein factor would be commonly used for animal cloning.

### 1.4. 3. Others

In addition to electrical activation and sperm protein factor, other agents such as ethanol, Ca-ionophore, $SrCl_2$ and ionomycin have been used for oocyte activation in different species. For example, ethanol was reported to stimulate activation of a number of oocytes. It is able to promote a rapid potentiation of $InsP_3$ –mediated $Ca^{2+}$ release through stimulation of $InsP_3$ production (Ilyin and Parker, 1992). A short incubation ranged from 3-10 minutes in the presence of 7% ethanol could activate approximately 83.4% of cow (Minamishi *et al.*, 1993), 22% of pig (Didion *et al.*, 1990), and 95% of sheep (Loi *et al.,* 1998) oocytes. Kim *et al* (1996; 1997) and Cha *et al* (1997) demonstrated that 15~16% of pig oocytes activated by 7% ethanol could develop to the blastocyst stage. In contrast, over 30% of bovine oocytes could develop to the blastocyst stage after activation by ethanol. However, ethanol and $SrCl_2$, which are successfully used in mouse oocytes, seem not adequate for activating oocytes from farm animals compared to ionomycin. Additionally, there is little reported information on strontium activating pig oocytes. Actually, most research on $Sr^{2+}$ activation has been done in mice (Bos-Mikich *et al.*, 1997; Kono *et al.,* 1996). Injection of $Sr^{2+}$ to mouse oocytes can induce activation rate at over 55% (Whittingham, 1980). Bos-Mikich *et al* (1997) exposured mouse oocytes to $Sr^{2+}$- containing medium for 2, 8 and 24 h, respectively. Interestingly, ethanol-activated embryos and those exposed to $Sr^{2+}$ for 2 h had significantly fewer inner cell mass (ICM) cells than the fertilised and those exposed to $Sr^{2+}$ for longer than 2 h. Yet, an increased number of ICM cells were observed in embryos exposed to $Sr^{2+}$ for longer than 2 h and also in the fertilised embryos. In

contrast, the number of trophectoderm cells was largest in ethanol-activated parthenote and smallest in fertilised embryos. However, postimplantation development was modestly improved by extended the time of exposure to $Sr^{2+}$– containing medium (Bos-Mikich *et al.*, 1997).

Recently, cloned offspring in different species such as pigs (Betthauser *et al.*, 2000) sheep (Loi *et al.*, 1998) cows (Wells *et al.*, 1999) were reported using ionomycin to activate nuclear transferred embryos. Compared to ethanol ionomycin seems to have advantages in activation rate and development competence, as well as reduction of damage during activation. Chemical activation does not require specific technical skills; also more cells can be activated at the same time. The conditions for activation are easily controlled. Thus, chemical activation would not be replaced by the other methods. To improve the efficiency of chemical activation and to reduce damage of the activated oocytes would enhance its application in either parthenogenetic activation or animal cloning.

## 1.5. Diploidisation of Activated Oocytes

An adequate electrical stimulation for parthenogenetically activated oocytes, or for cloned embryos should be measured by either blastocyst rate, or offspring born from cloned embryos. However, most parthenogenetically activated oocytes become haploid or polyploid, only small proportion of activated oocytes is diploid following activation. It has been confirmed that parthenogenetically activated diploid oocytes can develop much further than uniform haploid. Cytochalasin B, an alkaloid from fungi, which acts

on microfilaments, can be used for blocking the extrusion of the second polar body in activated oocytes so that the oocytes could become diploid. For example, Cha *et al* (1997) reported that pig oocytes treated with both electrical stimulation and cytochalasin B increased the incidence of diploid chromosome spreads, and accelerated development to the morula and blastocyst stage compared with the other treatment, which suggested a role of ploidy in the development of parthenotes. Also, Kim *et al* (1997) found that the percentage of pig oocytes with two female pronuclei was higher ($p<0.01$) in oocytes treated with cytochalasin B than that in ethanol treatment alone and that the average numbers of total cells and inner cell mass were significantly reduced ($p<0.05$) in the ethanol treatment alone as compared with the combined cytochalasin B and ethanol treatment. These results suggested that the ploidy may affect blastocoele formation and cell allocation to inner cell mass and trophectoderm in the pig. In addition, cytochalasin B must be dissolved in dimethyl sulphoxide (DMSO) that is toxic to oocytes when temperature is over 5°C. Perhaps, due to this reason, activated oocytes are cultured in 5–10 μg/ml cytochalasin B for 4 h normally over 50% of activated oocytes could become the diploid. Thus, it is relevant that using cytochalasin B to create more diploid oocytes after activation is essential for achieving a high rate of blastocysts.

Like cytochalasin B, 6-dimethylaminopurine (6-DMAP), a kinase inhibitor can also suppress the formation of a second polar body in all activated oocytes. Virtually all oocytes contain a single pronucleus with no polar body except mouse oocytes, whereas cytochalasin B treated oocytes normally contain two female pronuclei. However, it is postulated that 6-DMAP inhibits Mitogen activated protein (MAP)-kinase with a

consequent disruption of spindle organisation in metaphase II oocytes and irregularities in the gross morphology of the oocytes. Furthermore, 6-DMAP seems to enhance the speed of pronuclear formation and to suppress polar body extrusion. In addition, activated oocytes are cultured in 5 μM 6-DMAP for 3 h, whereas cytochalasin B needs at least for 4 h. For instance, Loi *et al* (1998) showed that cloned sheep embryos activated by the ionomycin-6-DMAP protocol could develop to term. In addition, they obtained the high rate diploidy (~ 90%) after ovine oocytes were incubated for 3 h in 6-DMAP following either ionomycin or ethanol activation. It is seemed that 6-DMAP would have some benefit to the development of the activated oocytes.

In general, although uniform haploid eggs can develop to the blastocyst stage, they will die shortly. Therefore, diploidization of activated oocytes is essential to improve their developmental competence.

## 1.6. *In Vivo* Developmental Competence of Parthenogenetic Pig Oocytes

The pig oocyte can be activated by a variety of different methods and agents. Oocytes activated by different methods and agents, in culture can develop to the blastocyst stage. However, these activated oocytes have by no means equally *in vivo* developmental competence when they are transferred into animals, as some oocytes do not have normal karyotypes such as haploidy usually seen in parthenogenetic embryos. In addition, some oocytes could be damaged due to an inadequate stimulation at activation. Therefore they normally have a small number of cells that cannot meet the

requirement for normal development. In most cases, they lack inner cell mass that will form fetus (Kim *et al.*, 1997). Previously, Kaufmann & Sachs (1975) observed that haploid mouse embryos clearly had a reduced total number of cells in the blastocyst stage. Jolliff and Prather, (1997) electrically activated pig oocytes that had been matured *in vitro* for 36 and 48 h, transferring them to animals, They found 10% parthenogenetic embryos would be able to develop to the elongated filamentous stage, which was equivalent to day 14 of gestation. More recently, Kurebayashi *et al.*, (2000) derived parthenogenetic fetuses on day 29. These results show that electrically activated porcine fetuses from *in vitro*-matured oocytes are able to develop *in vivo* for 29 days and the morphology of the fetuses collected looks normal compared to the fetuses collected from mated pigs at the same stages, but they are smaller in the mean crown-rump length. This demonstrates for the first time that parthenogenetic IVM pig oocytes are able to develop to the implantation stage. However, it has been well known that parthenogenetic embryos developing *in vivo* must die at some time point due to lack of the imprinting genes. How far can parthenogenetic pig embryos develop *in vivo*? This question is still waiting to be answered. The answer to this question would help us study gene imprinting in pigs and improve pig oocyte activation.

## 1.7. Objectives of This Project

This project was originally based on *in vitro* culture systems including *in vitro* maturation of pig oocytes and *in vitro* culture of parthenogenetic embryos. Thus, the establishment of an *in vitro* culture system was more important for subsequent studies

on pig oocyte activation although some ovulated oocytes were also used for this project later.

The aims of this project were

1. To establish an *in vitro* culture system for either pig oocyte maturation or embryo culture;
2. To investigate interactions across oocyte age, voltages, the number of pulses and pulse duration as well as activation conditions including the effects of oocyte maturation, activation media, $Ca^{2+}$, $Mg^{2+}$ in activation and diploidization, optimising the electrical activation protocol for *in vitro* matured pig oocyte.
3. To examine the *in vivo* developmental competence of IVM pig oocytes activated by the improved protocol.
4. To use the improved activation protocol for pig nuclear transfer.

# Chapter 2

# General Materials and Methods

## 2.1. Ovary Collection and Preparation

Ovaries were obtained from a local abattoir and maintained in a box at 20-30°C during transportation to the laboratory (1-2 h). When ovaries arrived, temperature of the ovaries was measured, if the temperature were below 20°C, the ovaries would not be used. Ovaries were transferred into a plastic beaker and rinsed three times with 1000 ml of PBS (Phosphate-Buffered Saline; Unipath Ltd., Basingstoke, UK) that had been warmed up in a water bath at 28-30°C. They were then kept in the water bath at 30°C for aspiration.

## 2.2. Oocyte Preparation and Maturation *In Vitro*.

Cumulus oocyte complexes (COCs) were aspirated from follicles 3-8 mm in diameter using a 10 ml syringe fitted with an 18 G needle. The aspired follicular fluid was stored in 50 ml sterile universal containers (Nunc, Rosklide, Denmark) and kept in the water bath. After collection, the follicular fluid was removed from the containers. Collected samples were washed three times with PVA-TL-HEPES medium and then put in about 15-20 ml of the same medium in a 90-mm dish (Sterilin Ltd, Stone, Staffs UK). The dish was placed on a warm stage at 37°C digitally displayed under a microscope (Wild Heerbrugg M8, Switzerland). COCs were searched and selected. Only COCs with uniform ooplasm and at least three layers of compact cumulus cells were

transferred to another 35-mm petri dish (Nunc, Rosklide, Denmark) for maturation (Fig.2.1 and 2.2). It must be noted that all the media used should be warmed up to 30–37 °C before use.

Preparation of porcine follicular fluid was to collect supernatant from the universal containers containing porcine follicular fluid. The supernatant was put in centrifugal tubes and spun at 3,000 rounds per min (RPM) for 30 min. Supernatant was taken from the tubes and aliquoted to 1 ml in 1.5 ml eppendorf tubes. The aliquoted tubes were stored in a freezer at –20°C until use.

Selected oocytes were washed three times in North Carolina State University 23 (NCSU 23) medium + 10% porcine follicular fluid (pFF) that had been equilibrated in a 5% $CO_2$ incubator at 39°C for at least 2 h. 500 µl drops of maturation medium, which was composed of NCSU 23 + 10% pFF + 1% essential amino acids (B-6766, Sigma) + 0.1% nonessential amino acids (M-7145, Sigma), were made up in 4 well dishes (Nunc, Denmark) and supplemented with 10 IU/ml eCG (Intervet, Ireland Ltd) and 10 IU/ml hCG (Intervet, Ireland Ltd) before transfer of COCs in the dishes. Selected COCs, in-groups of 50 COCs, were transferred into 4-well dishes (50 COCs/500 µl) by a glass pipette with a minimum amount of the maturation medium in order to avoid diluting the hormones. The dishes were then placed in the incubator for 22 h. At the end of culture of 22 h, COCs were washed three times with fresh, equilibrated NCSU 23 medium + 10% pFF + amino acids (Fig.2.3.) and transferred into the same medium without hormonal supplements, cultured at 39°C in 5% $CO_2$ for an additional 22 h period (Fig.2.4).

**Fig 2.1**. Cumulus – Oocyte – Complexes (COCs) aspirated from ovarian follicles.

**Fig 2.2.** Pig oocytes with compact layers of cumulus cells selected for *in vitro* maturation

**Fig 2.1.**

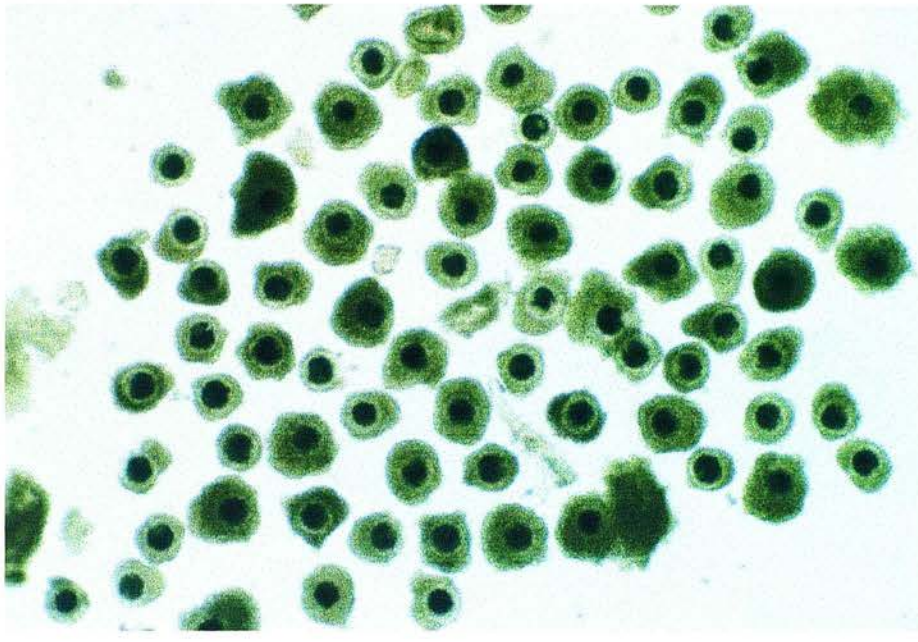


**Fig 2.2.**

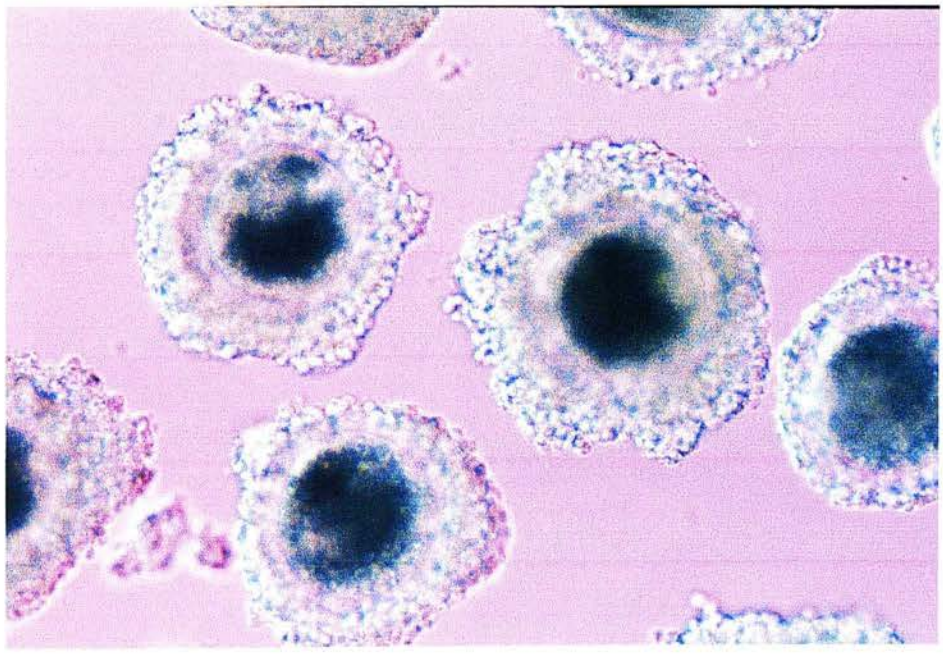


**Fig 2.3.** One oocyte matured in NCSU 23 medium with 10% pFF and hormones for 22 h. The picture shows expanded cumulus cells.

**Fig 2.4**. This oocyte matured in NCSU 23 medium with 10% pFF for 44 h. The cumulus expanded and some connections among cumulus cells were lost.

**Fig 2.3.**

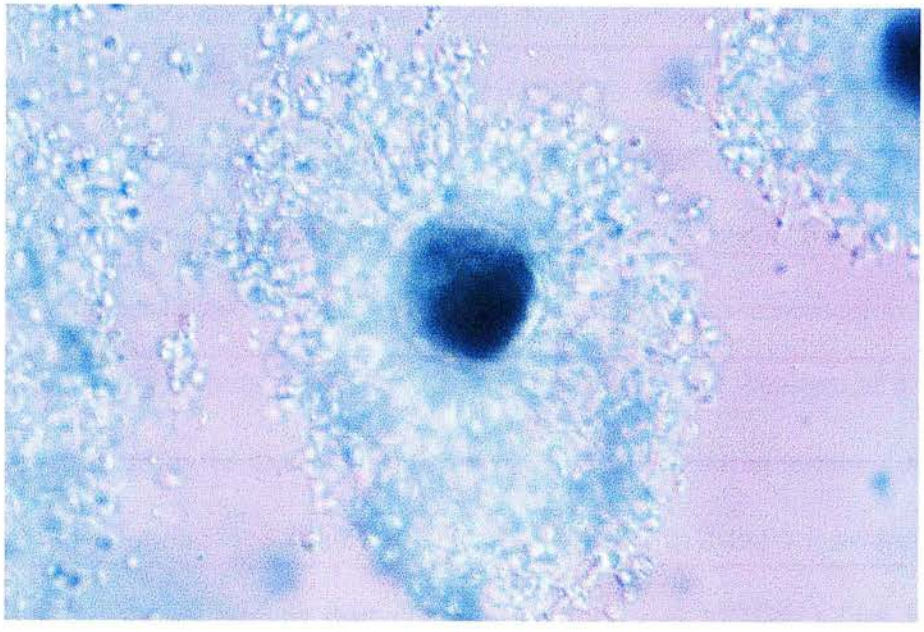
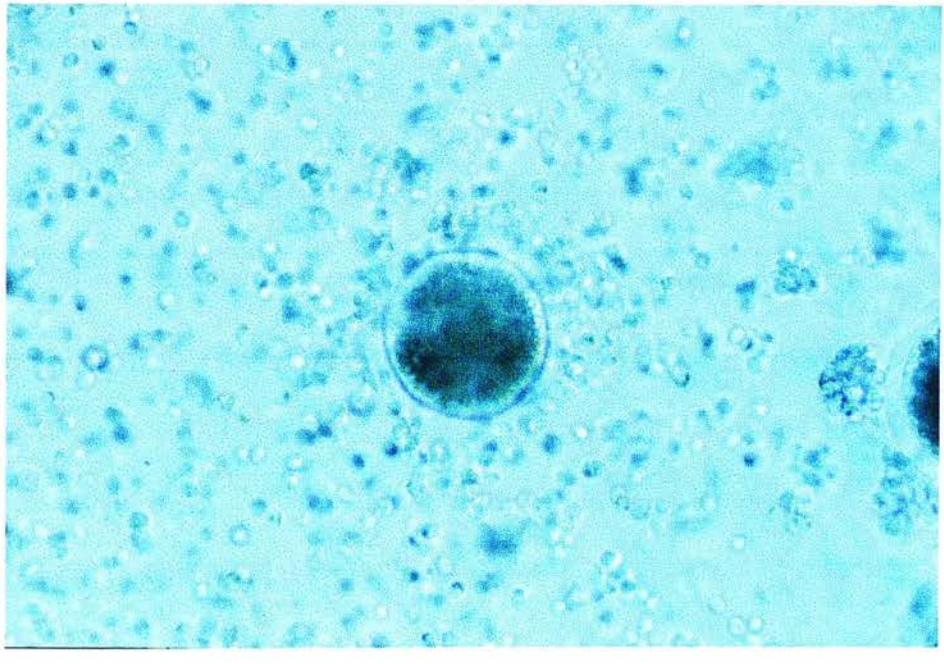


**Fig 2.4.**



## 2.3. Preparation and Setting of the Activation Machine

Activation machine **(Model CF-150/B series 981193 230 V 50/ 60 Hz, made in Hungary)** is turned on half-hour before use, and its parameters and connection (OUT seen in Figs 2. 5, 2.6 and 2.7) with the chamber must be checked carefully and repeatedly in order to make sure that it is in a good connection with the machine. The parameters for activation should be reset if they are not correct. Repeat representing numbers of pulses should be set by pressing the Button (1) to 3, electrical field strengths e should be set by turning the Button (2) to 20 at MODE (1), because of a gap of 200 μm between wires in the chamber (1 kV/cm DC). The duration of stimulation should be set by turning Button (3) to 80 μsec at MODE 2 (MODE 1, 2 and 3 from top to bottom) AC should be set by turning Button (4) to 5 voltages at MODE 3 (see Figure 2.5). Following resetting, check all the settings again by pressing "MODE" in order to make sure that they are really correct.

## 2.4. Preparation and Activation of Porcine Oocytes

After 44 h of maturation, oocytes surrounded by expanded cumulus cells were denuded mechanically by repeated pippetting (Figs 2.8 and 2.9). Denuded oocytes were selected and washed twice in $Ca^{2+}$ free, Hepes–buffered NCSU 23 medium and activation medium (0.3 M Mannitol + 0.1 mM $Mg^{2+}$ + 0.05 mM $Ca^{2+}$), respectively. Oocytes were mounted between electrodes 200 μm apart covered with 0.2-ml drop of activation medium in a chamber connecting with a fusion machine (CF-150/B series,

**Fig 2.5. The front of the fusion machine**
**Buttons (1) of pulse number**

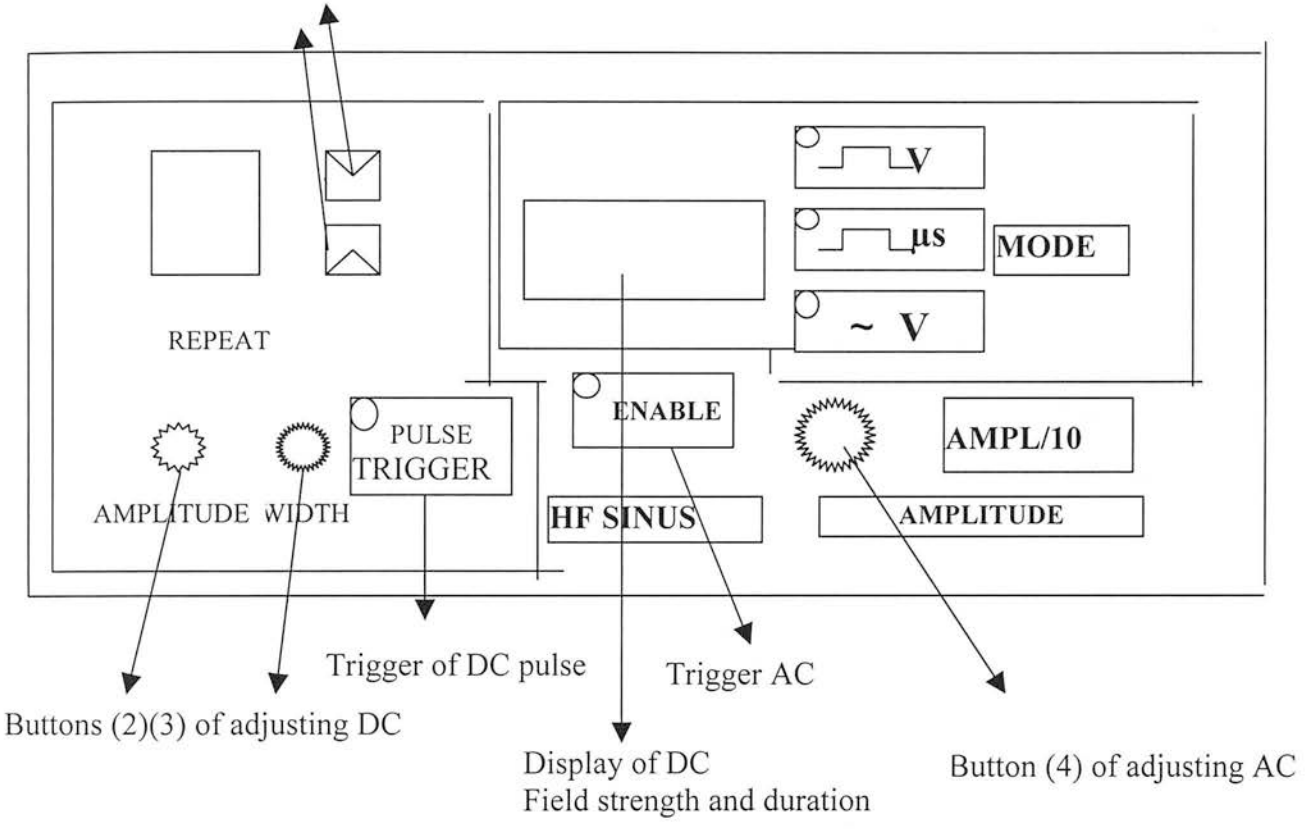



**Fig 2.6. The back of the fusion machine**

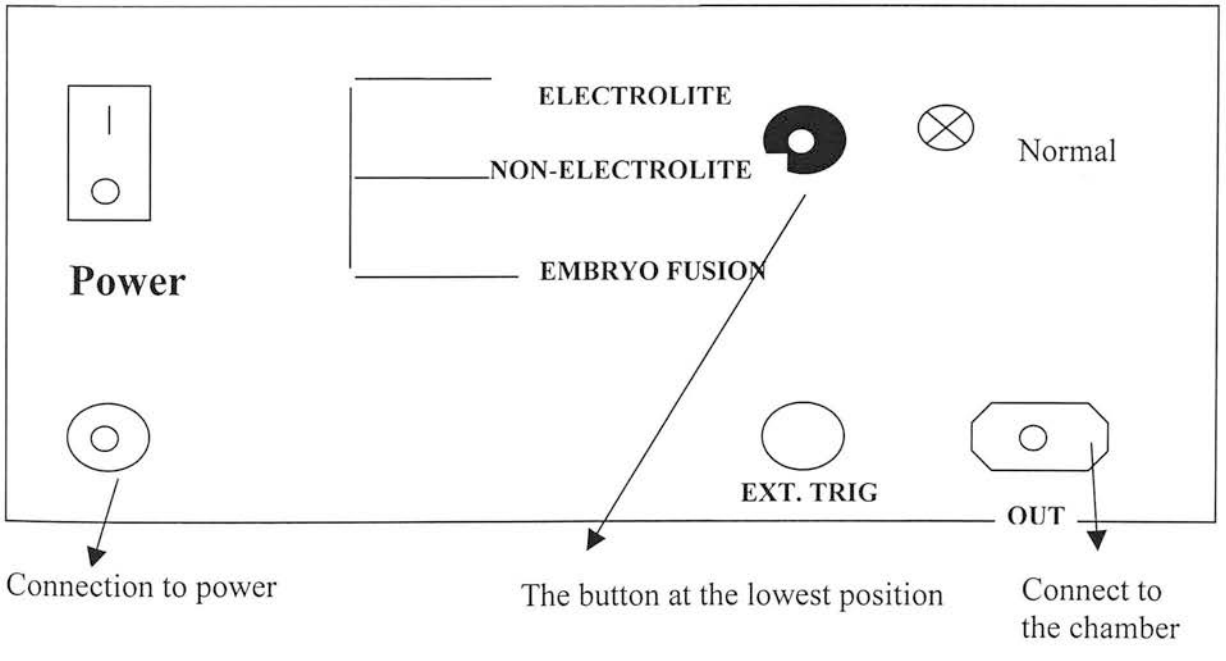
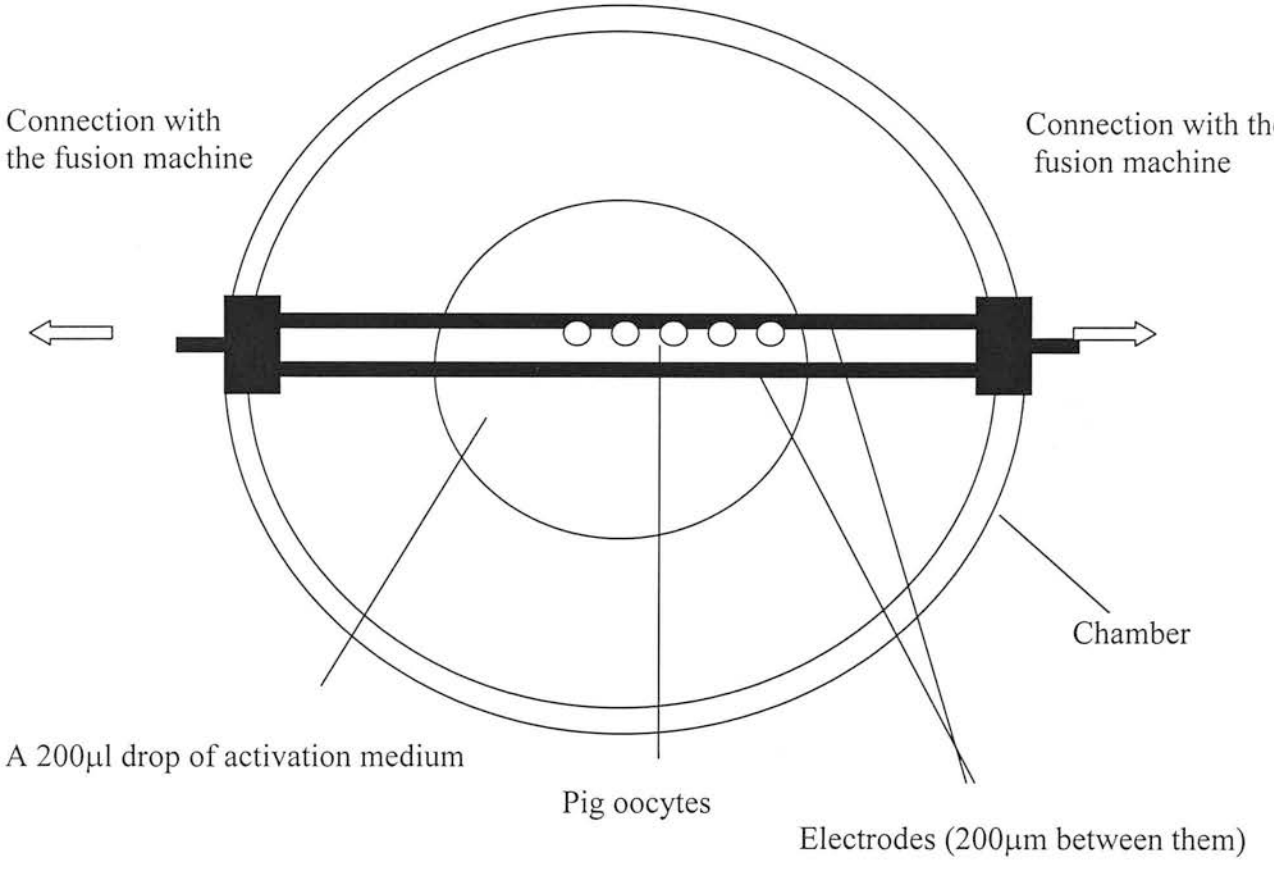


**Fig 2. 7 Activation chamber**



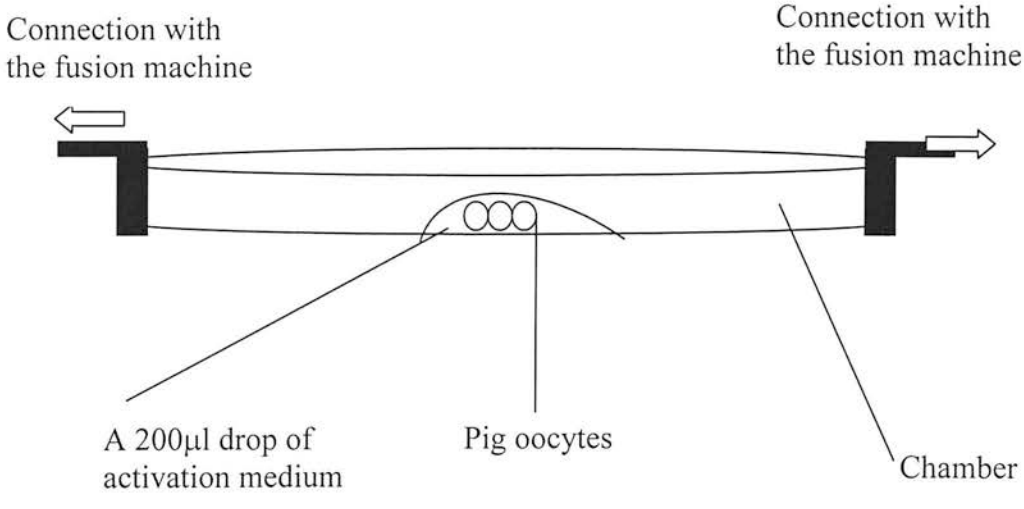



**Fig 2.8.** Oocytes denuded by repeated pippetting. The picture shows two denuded oocytes after 44-h maturation.

**Fig 2.9.** The picture shows one denuded oocytes with a visible first polar body pointed by the arrow.

**Fig. 2.8.**

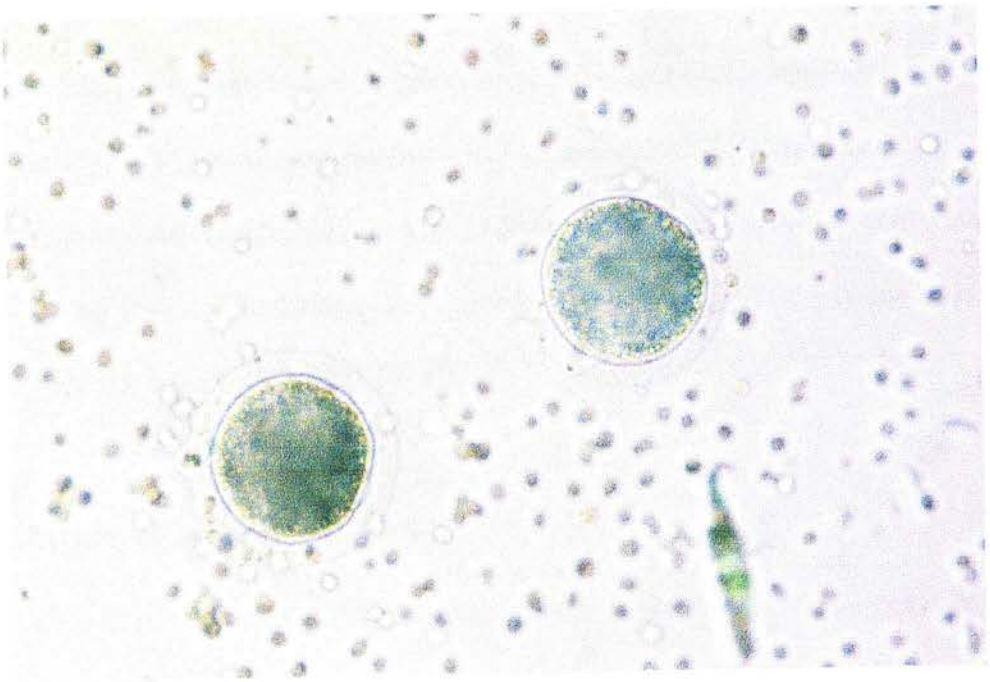
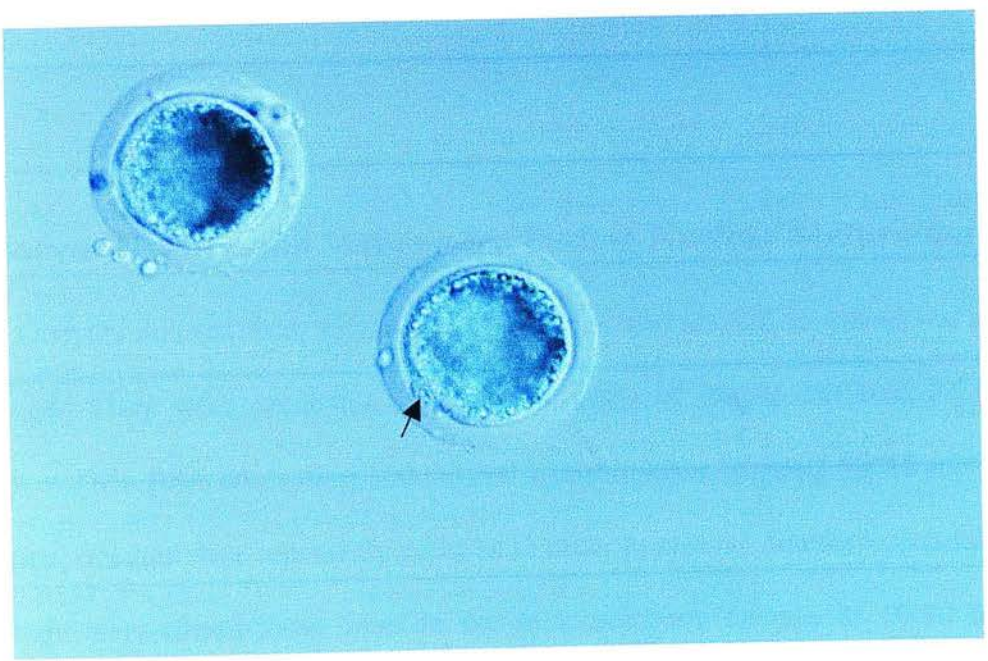


**Fig. 2.9.**



BLS-Ltd, Hungary). Then, they were immediately activated by 3 x 80 μsec. consecutive pulses of 1.0 kV/cm DC following 0.25 kV/cm AC for 5 sec. Activation medium in the chamber was changed every time when the next batch of oocytes needed to be activated. Also, after washing media were used three times, they needed to be changed, too. After electrical activation, oocytes were washed in NCSU 23 medium + 0.4% BSA (A6003, Sigma) and transferred into 500 μl drops of the same medium in 4-well dishes containing 7.5 μg/ml cytochalasin B (C-6762) (30-40 oocytes/500 μl) and cultured in the incubator for 6 h, at 39 °C, 5% $CO_2$ in air.

## 2.5. Culture of Activated Oocytes

After 6 h of cytochalasin B treatment, oocytes were rinsed three times in NCSU 23 medium + 0.4% BSA and transferred into 500 μl drops of NCSU 23 medium + 0.4% BSA (A6003 or A8022) in 4-well dishes covered with mineral oil (M-8410, Sigma) (30-40 oocytes/500 μl). Subsequently, oocytes were cultured at 39°C, 5% $CO_2$ in air for 6-7 days.

## 2.6. Oocyte Fixation and Staining for Testing Nuclear Maturation

Oocytes derived from maturation were fixed and stained to assess the nuclear maturation. They were incubated in 50 μl droplets of Hepes–buffered NCSU 23 medium + 0.4% BSA containing 300units/ml hyaluronidase (Sigma) for 10 min. After incubation, oocytes were repeatedly pipetted in order to remove attached cumulus cells. Then, they were rinsed three times in the same medium. Groups of 10-30 oocytes

completely denuded from cumulus cells were mounted on previously cleaned glass slides. A coverslip was attached using a small amount of a mixture of solid paraffin wax (4%) and Vaseline (96%) applied to two of its edges. Whilst observing the oocytes, the coverslip was gently pressed down until the oocytes were not able to roll but intact. The mounted slides were very gently placed in a jar containing 40 ml of freshly made fixation medium–methanol: acetic acid (3: 1).

Oocytes were left in the fixative solution for a minimum time of 72 h, and then stained with 1% orcein (O-7380, Sigma). For preparation of orcein solution, 1 g of orcein was completely dissolved in 45 ml of acetic acid on a heater. After cooling, 55 ml distilled water was gently added and the suspension filtered through a filter paper (11 μm pore size) (Whitman International Ltd. Maidstone, UK). Prior to use the acetic–orcein was refiltered.

For staining, individual slides were removed from the fixation medium and placed under a dissecting microscope. The oocytes were examined to make sure that oocytes were still attached on the slide. Subsequently, the slide was cleaned with a filter paper, 1% orcein solution was slowly drawn into the oocyte compartment by using a 200μl pipette. After drying for 5 min at room temperature, the orcein was washed by gentle flushing with a solution containing water: acetic acid: glycerol (3: 1: 1). Then, the slides were immediately observed or stored in a fridge at 4°C until use.

## 2.7. Blastocyst Staining with Hoechst 33342

Day 6 or day 7 blastocysts were washed twice in Hepes-buffered NCSU 23medium + 0.4% BSA; they were then transferred into 50 μl droplets of the same medium containing 5 μg/ ml Hoechst 33342 (Sigma) and cultured in the incubator for 10 min. After incubation, blastocysts were gently mounted on a cleaned glass slide and covered with a cleaned glass coverslip. The slides were immediately observed under UV light or stored in a fridge at 4°C until observation. It must be noted that the slides should not be exposed to lights or UV lights for a long time, otherwise colour would fade quickly.

## 2.8. Karyotyping Porcine Blastocysts

A normal porcine mitotic karotype consists of 38 chromosomes (2N). Day 6 or 7 porcine expended blastocysts were cultured in NCSU 23 medium + 0.4% BSA containing 0.1 μg/ml Colcemid (D-7385, Sigma) for at least 6 h in order to synchronise the divisions of the blastocysts (at the Metaphase). Six hours later, the blastocysts were taken out of the incubator and transferred into a hypotonic solution of 1% sodium citrate (S-4641, Sigma) and left in the medium for 5 min. Subsequently, a single blastocyst was placed in a marked frame on the middle of a freshly cleaned glass slide; medium surrounding the blastocyst was gently dried by wiping with a small piece of filter paper. The blastocyst was then immediately flushed by slowly dropping one by one drops of fresh fixation medium composed of 3 parts of methanol (M/4000/17, Fisher) and 1 part of acetic acid (BDH) at room temperature, meanwhile keeping watching the blastocyst under a microscope. If its zona has broken and the chromosomes spread already, the

slide was marked with information on the blastocyst such as date, treatment and so on, and left on the bench to dry. Individual blastocyst was stained in 5% Giemsa at pH 6.8 for 10–15 min, at room temperature. Afterwards the slides were washed by distilled water and dried in air at room temperature. The chromosome content of each spread was determined by observation under a microscope (Nikon microphot-SA, Japan).

# Chapter 3

# Optimisation of an electrical activation protocol for porcine oocytes

## 3.1. Introduction

Recently, several teams in the world cloned piglets from somatic cells (Polejaeva *et al*; 2000; Onishi *et al* 2000; Betthauser *et al*; 2000). However, the first cloned piglets were produced by a serial nuclear transfer that could bypass the inefficiency of the current activation procedures (Polejaeva *et al*; 2000). Subsequently, a piglet was cloned by a combination of direct injection of fetal fibroblast cell with delayed activation by electrical stimulation. The same activation method was also used to activate ovulated oocytes as a control, 31.2% oocytes developed to the blastocyst stage, whereas only 2.4% when using *in vitro* matured (IVM) porcine oocytes (Onishi *et al*; 2000). Cloned piglets with *in vitro* matured oocytes as recipient cells were created by activating cloned embryos with Ionomycin. In the control, only 23% of IVM sow oocytes that were activated by Ionomycin developed to the blastocyst stage (Betthauser *et al*; 2000). Based on these experiments, it seems that poor oocyte activation may contribute to the inefficiency of pig nuclear transfer. In other words, poor oocyte activation in the pig remains unresolved although cloned piglets were produced. In addition, most reports have shown that activation rate increases with ageing of the oocytes in pigs [Wang *et al* 1999; Machaty *et al*, 1999; Liu and Moor 1997, Mates *et al*, 1995). In contrast to

these data, Jolliff and Prather (1997) did not obtain a similar result. Moreover, it is thought that the rate of activation of oocytes would be greatly improved with the use of multiple pulses, since multiple electrical pulses presumably cause repeated calcium transients, which induce activation in oocytes not capable of responding to a single pulse (Collas *et al*, 1993). However, a single pulse is still more commonly used in most activation protocols for pig oocytes [Onishi *et al*; 2000, Jolliff and Prather, 1997; Koo *et al*, 2000, Kure-bayashi *et al* 1996). Therefore, the aim of these experiments in this chapter was to establish an efficient protocol for pig oocyte activation meanwhile I focused on investigating factors influencing the efficiency of electrical activation in pig oocytes and the interactions across parameters such as oocyte age, field strength, pulse number and pulse duration.

Three interrelated series of experiments were carried out. The objective of the first study was to investigate the effects of epidermal growth factor (EGF) and amino acids in maturation medium on pig oocyte activation in order to establish an *in vitro* maturation system for pig oocyte activation. In the second study, electrical parameters were optimised by investigating the interactions across time of oocyte maturation, field strength, pulse number and pulse duration. In the third study, blastocysts produced by the improved activation protocol were karyotyped to demonstrate their developmental potential.

## 3.2. Materials and Methods

### 3.2.1. Ovary collection and oocyte maturation

Gilt ovaries were collected from a local abattoir and kept in a box at 25-30 °C during transportation. When ovaries arrived at the laboratory, they were put in a

beaker and washed three times with warm PBS, then stored in a water bath at 25-30°C for use.

Cumulus oocyte complexes (COCs) were aspirated from ovarian follicle 3-8 mm in diameter with a 10-ml syringe fitted with an 18G needle. Follicular fluid was stored in 50 ml universal containers and left on the bench for 5 min at 25-30°C. Subsequently, COCs were washed three times with Hepes-buffered TALP medium (TL-Hepes) containing 0.1% polyvinylalcohol (PVA). Only COCs with uniform cytoplasm and at least three layers of compact cumulus cells were selected for maturation. Maturation medium was bovine serum albumin (BSA)-free NCSU 23 medium (Peters & Wells, 1993) supplemented with 10% (v/v) porcine follicular fluid (pFF), 0.6 mM cysteine, 10 IU/ml eCG (Intervet UK Ltd, Cambridge UK) and 10 IU/ml hCG (Intervet UK Ltd, Cambridge UK), 1% essential-amino acids (v/v) (B-6766, Sigma) and 0.5% non-essential amino acids (v/v) (M-7145, Sigma). Fifty selected COCs were transferred to 500 µl drops of maturation medium in 4-well tissue culture dishes (Nunc, Denmark), which had been equilibrated in an incubator for at least 2 h at 39°C, 5% $CO_2$ in air, then cultured for 22 h in the same conditions. After 22 h of maturation, the COCs were washed three times with fresh, warmed maturation medium without hormonal supplements, then transferred into 500 µl drops of the same medium and cultured for an additional 22 h period.

### 3.2.2. Oocyte activation

At the end of 44 h-culture, oocytes were stripped of cumulus cells on a warm stage at 37°C by repeated pipetting; only denuded oocytes were selected for activation. Denuded oocytes were washed three times in $Ca^{2+}$ free–TL-Hepes-PVA

medium, and then rinsed twice in activation medium (0.3 M Mannitol, 0.1 mM $Mg^{2+}$ and 0.05 mM $Ca^{2+}$). Oocytes were transferred into a 0.2 ml drop of activation medium in a chamber connected with a fusion machine (FC-150, BLS Ltd, Hungary) and activated by 3 x 80 μsec. consecutive pulses of 1.0 kV/cm DC following 0.25 kV/cm AC for 5 sec. Subsequently, they were immediately transferred into a 500 μl drop of NCSU 23 medium containing 0.4% BSA (A 6003, Sigma) and 7.5 μg/ml cytochalasin B (CB) (C-6762, Sigma) and cultured in the medium for 6 h in the incubator at 39°C in an atmosphere of 5% $CO_2$ in air.

### 3.2.3. Culture of activated oocytes

After 6 h of CB treatment, activated oocytes were washed three times in fresh NCSU 23 medium supplemented with 0.4% BSA, 30-40 activated oocytes were transferred into a 500 μl drop of the same medium containing 0.4% BSA and cultured in 4-well dishes covered with mineral oil (M - 8410, Sigma) for 6 or 7 days at 39°C, 5% $CO_2$ in air.

### 3.2.4. Nuclear staining of porcine oocytes

Oocytes derived from maturation were fixed and stained to assess the nuclear maturation. They were incubated in 50 μl droplets of Hepes–buffered NCSU 23 medium containing 0.4% BSA and 300 units hyaluronidase (Sigma) for 10 min. After incubation, oocytes were repeatedly pipetted in order to remove attached cumulus cells. Groups of 10-30 oocytes completely denuded from cumulus cells were mounted on cleaned glass slides. A coverslip was attached using a small amount of a mixture of solid paraffin wax (4%) and Vaseline (96%) applied to two

edges of the slide. Whilst observing the oocytes, the coverslip was gently pressed down until the oocytes were not able to roll but intact. The mounted slides were very gently placed in a jar containing 40 ml of freshly made fixation medium–methanol: acetic acid (3: 1).

Oocytes were left in the fixative solution for a minimum of 72 h, and stained with 1% orcein (O-7380, Sigma). Then, the slides were observed immediately or stored in a fridge at 4°C until observation.

### 3.2.5. Staining of nuclei in parthenogenetic blastocysts.

Day 6 or day 7 blastocysts were washed twice in Hepes-buffered NCSU 23 with 0.4% BSA, they were then transferred into 50 μl droplets of the same medium containing 5 μg / ml Hoechst 33342 (Sigma) and cultured in the incubator for 10–15 min. After incubation, blastocysts were gently mounted on a cleaned glass slide and covered with a cleaned glass coverslip. The slides were immediately observed under UV light or stored in a fridge at 4°C until observation.

## 3.3. Experimental Design

### 3.3.1. Study 1. Effects of EGF and amino acids in maturation medium

Maturation media used were (1) NCSU 23 medium, 0.6 mM cysteine, 10% pFF as a control; (2) NCSU 23 medium, 0.6 mM cysteine, 10% pFF and 10 ng/ml EGF; (3) NCSU23 medium, 0.6 mM cysteine, 10% pFF, 1% essential amino acids and 0.5% non-essential amino acids (AA) (v/v); (4) NCSU 23 medium, 0.6 mM cysteine, 10% pFF, 10 ng/ml EGF, and AA. COCs were matured respectively in

these four media with hormonal supplements for 22 h, then cultured in the same medium without hormonal supplements for an additional 22 h period. Subsequently, oocytes were stripped of cumulus cells by repeated pipetting. Denuded oocytes were randomly divided into 4 groups and activated in the activation medium by 3 x 80 μsec. consecutive pulses of 1.25 kV/cm DC following 0.25 kV/cm AC for 5 sec, and treated in 7.5μg/ml cytochalasin B for 5 h. Then, activated oocytes were cultured in NCSU23 medium with 0.4% BSA for 6 days at 39°C, 5% $CO_2$ in air. The experiments were repeated 5 times. Each treatment contained a total number of 231-263 oocytes.

In addition, COCs were matured in NCSU 23 medium supplemented with 10% pFF, 0.6 mM cysteine and AA in 10 dishes for 0, 22, 36, 37, 38, 39, 40, 41, 42 and 43 h, respectively. Oocytes randomly taken from one of 10 dishes at each of time points were fixed in acetic acid : methanol (1:3) for 3 days, then they were stained with 1% orcein. Stained oocytes were assessed by standard morphological criteria.

### 3.3.2. Study 2. Optimisation of activation parameters including time of oocyte maturation, field strength, numbers of pulses and pulsing duration for oocyte activation

#### 3.3.2.1. *Experiment A: Interaction of timing of oocyte maturation and field strengths.*

Oocytes matured for 36, 40, 44 and 48 h were activated by a single pulse of 1.0, 1.25 and 1.5 kV/cm DC for 80 μsec. respectively, following 0.25 kV/cm AC for 5 sec. (4 x 3 factorial design). Activated oocytes were cultured in 7.5 μg/ml

cytochalasin B for 6 h, then cultured in NCSU23 medium with 0.4% BSA for 6 days. Embryonic development was assessed by blastocyst formation and cell number counting. The experiment was repeated three times with a total number from 89 to 200 oocytes in each of the treatments.

#### 3.3.2.2. *Experiment B: Interaction of field strengths and number of pulses*

Oocytes matured for 44 h were randomly divided into 9 groups and activated by 1, 3 and 5 x 80 μsec. consecutive pulses of 1.0, 1.25 and 1.5 kV/cm DC following 0.25 kV/cm AC for 5 sec (3 x 3 factorial design), respectively. Activated oocytes were treated in CB for 6 h, then cultured in NCSU23 medium with 0.4% BSA for 7 days. Each treatment had a total number of over 100 oocytes in three replicates. Embryonic development was assessed by blastocyst formation and cell number counting of the blastocysts.

#### 3.3.2.3. *Experiment C: Interaction of number of pulses and duration of pulse(s).*

44 h-post matured oocytes were activated with 0.25 kV/cm AC for 5 sec followed by 1 or 3 consecutive pulses of 1.0 kV/cm DC for 20, 40, 60, 80, 100, 120, 140 and 160 μsec. (2 x 8 factorial design), respectively. Activated oocytes were treated in CB for 6 h, then cultured in NCSU 23 medium with 0.4% BSA for 7 days. The experiment was repeated 7 times with a total number ranging from 233 to 260 oocytes in each of the treatments. Also, embryonic development was assessed by the method mentioned above.

### 3.3.3. Study 3: Karyotype of parthenogenetic blastocysts

Day 6 or 7 blastocysts produced by the improved activation protocol were washed in fresh, warmed NCSU 23 medium with 0.4% BSA. They were cultured in 500 µl NCSU 23 medium + 0.4% BSA containing 0.2 µg/ml Colcemid (Life Technologies) for at least 6 h in order to synchronise the divisions of the blastocysts. Six hours later, the blastocysts were taken out of the incubator and transferred into a hypotonic solution of 0.6% sodium citrate (S-4641, Sigma) and left in the medium for 5 min. Subsequently, a single blastocyst was placed in a marked frame on the middle of a freshly cleaned glass slide. Then, the blastocyst was immediately flushed by dropping fresh fixation medium composed of 3 parts of methanol (M/4000/17, Fisher) and 1 part of acetic acid (BDH) at room temperature and left on the bench to dry. Individual blastocyst was stained in 5% Giemsa at pH 6.8 for 10 – 15 min. Thereafter, slides were washed by distilled water and dried in air at room temperature. The chromosome content of each spread was determined by observation under a microscope (Nikon microphot-SA, Japan). Meanwhile *in vivo* fertilised and *in vitro* cultured blastocysts were also karyotyped in the same way as controls. The normal karyotypes (2n=38) of the blastocysts were determined by counting the number of spread chromosomes. A total of 130 parthenogenetic blastocysts were karyotyped in this experiment, meanwhile 22 *in vivo* fertilised blastocysts were also karyotyped as other controls.

## 3.4. Statistical Analysis

All the data were analysed by Excel, Student's T- test and Chi-square test. A probability of $p < 0.05$ was considered to be statistically significant.

## 3.5. Results

### 3.5.1. Study 1: Effects of EGF and amino acids in maturation medium

The effects of EGF and amino acids on the development of activated porcine oocytes are shown in Table 3.1. The maturation medium containing AA alone gave the highest blastocyst rate of 32.1% (80 blastocysts/249 used oocytes), which significantly differed from the media containing EGF (20.2%; 53/263 in medium with EGF alone and 25.5%; 59/231 in medium with EGF and AA) ($p<0.05$; T-test.), but not differed from 26.2% (61/233) ($p>0.05$) in the controls. Moreover, no differences across the treatments were observed in the mean number of nuclei/blastocyst ($p>0.05$).

In addition, 82% oocytes at 0 h of maturation were at the GV stage, whereas 68% oocytes in NCSU 23 medium supplemented with 10% pFF and AA reached the MI stage at 22 h of maturation. However, the percentage of MII oocytes was 75% at 36 h, over 90% from 37 to 43 h (Fig.3.1).

### 3.5.2. Study 2. Optimisation of electrical parameters including oocyte age, field strengths, pulse number and pulse duration.

#### 3.5.2.1. Interaction between time of oocyte maturation and field strength

In this experiment, blastocyst rates in all treatments were increased with oocyte ageing during a period from 36 to 44 h of maturation (Fig.3.2). For example, in 1.0 kV/cm treatment, the blastocyst rate at 36 h was only 14.5% (16/110 oocytes), whereas 18.9% (34/180) at 40 h and 29.1% (43/148) at 44 h, respectively. A similar

result was also observed at 1.25 kV/cm, the blastocyst rates were 20.9% (18/86), 20.1% (38/189) and 35% (70/200) at the same time points. Likewise, at 1.5 kV/cm, the blastocyst rates of 17.3% (17/98), 27.4% (43/157) and 41.1% (67/163) at 36, 40 and 44 h were obtained. There was a linear relationship between time of maturation and blastocyst rate for two of the three treatments during a period from 36 to 44 h of maturation regardless of the effect of field strength. However, the blastocyst rate at 48 h dropped from 35 to 18% at 1.25 kV/cm and from 41.1 to 20.2% (24/119) at 1.5 kV/cm. By contrast, the blastocyst rate for 1.0 kV/cm still appeared an increase, reaching 32.3% (54/167). No differences across the treatments were found in the mean number of nuclei / blastocyst ($p>0.05$) (data not shown).

### 3.5.2.2. Interaction between pulse number and field strength

In this experiment, the interaction of pulse number and field strength is shown in Fig.3.3. Three x 80 μsec. consecutive pulses of 1.0 kV/cm yielded the highest mean blastocyst rate of 54.9% (75/138), which was significantly higher than those in the most other treatments ($p<0.05$). The data showed that the mean blastocyst rates in a single pulse of 1.0, 1.25 and 1.5 kV/cm were 32.3% (44/135), 36.8% (47/128) and 33.6% (45/135); whereas 54.9% (75/138), 26.1% (33/114) and 34.7% (41/115) in 3 consecutive pulses of 1.0, 1.25 and 1.5 kV/cm DC, respectively, compared with 40.5% (70/166), 26.8% (31/103) and 20.8% (34/159) in 5 consecutive pulses of 1.0, 1.25 and 1.5 kV/cm DC. Likewise, no differences across the treatments were observed in the mean number of nuclei/ blastocyst ($p > 0.05$) (the data not shown).

### 3.5.2.3. Interaction between pulse number and pulse duration

In this experiment, a comparison of 1 and 3 consecutive pulses with different pulse durations was made. The results are shown in Fig. 3.4. The mean blastocyst rates of the oocytes activated by one single pulse of 1.0 kV/cm DC for 20, 40, 60, 80, 100, 120, 140 and 160 μsec. were 21.7 (49 blastocysts/240 oocytes), 19.8 (47/237), 22 (49/235), 24.4 (60/244), 32.9 (74/233), 24.3 (56/235), 26.2 (61/233) and 24.7% (63/260), respectively, whereas 26.3 (58/234), 32.2 (73/233), 35.1 (80/236), 36.4 (84/237), 26.1 (61/236), 23.9 (56/239), 27.3 (62/234) and 28.8% (66/233) in the treatment with 3 pulses for the corresponding durations. In the single pulse treatment, the highest mean blastocyst rate of 32.9% appeared for 100 μsec. not significantly different from 36.4% in the treatment of 3 consecutive pulses for 80 μsec ($p>0.05$). Apparently, the mean blastocyst rates of oocytes activated by a single pulse of 1.0 kV/cm DC for 100 μsec. or by three pulses of the same field strength for 40, 60 and 80 μsec were comparable. By contrast, blastocyst rates in three pulses for 60, 80 μsec were significantly higher than those in a single pulse for the corespondent durations ($p< 0.05$ and $<0.01$).

### 3.5.3. Study 3: Karyotype of parthenogenetic blastocysts

Day 6 or 7 blastocysts were produced by 3 x 80 μsec. consecutive pulses of 1.0 kV/cm DC with 44 h post matured oocytes. A total number of 130 blastocysts were karyotyped. However, only 73 blastocysts had one–nine cells with spread chromosomes, 61 out of which were diploid (2N), 7 tetraploid (4N) and 5 mixed ploidy (only 2N mixed with 4N). The percentage of diploid blastocysts (61/73) was

83.6%, whereas 9 of 21 *in vivo* fertilised and *in vitro* cultured blastocysts had spread chromosomes, all of 9 were diploid (100%) (Table 3.2).

**Table 3.1. Effect of amino acids and epidermal growth factor (EGF) in maturation medium**

| Maturation medium | No. of oocytes (N) | No. of cleaved oocytes (N) | No. of blast.* | *Percentage of blast. (%) | No. of nuclei/blast. Mean ± SD** |
|---|---|---|---|---|---|
| NCSU23 | 233 | 172 | 61 | 26.2 | 23.6 ± 3.4 |
| NCSU23+EGF | 263 | 206 | 53 | 20.2[b] | 25.8 ± 5.0 |
| NCSU23+AA | 249 | 210 | 80 | 32.1[a] | 26.0 ± 4.4 |
| NCSU23+EGF+AA | 231 | 202 | 59 | 25.6[b] | 25.7 ± 4.9 |

There is a significant difference between *a* and *b*. ($p<0.05$; T-Test)
* The % blastocyst is based on total numbers of oocytes and blastocysts used but the t-tests were done on separate means for the different replicates.
Blast. = Blastocysts; ** SD = Standard Deviation.

**Fig.3.1.** A total number of 436 oocytes were matured in the maturation medium supplemented with AA in 10 dishes. Oocytes in one of the dishes were fixed and stained at each of time points, respectively. 82% of 50 oocytes at 0 h were at the GV stage, 68.2% of 44 at 22 h were at the MI stage. The percentage of MII oocytes at each of time points from 36 to 43 h was 75, 100, 97.5, 90.7, 97.3, 100, 93.1 and 100%, respectively

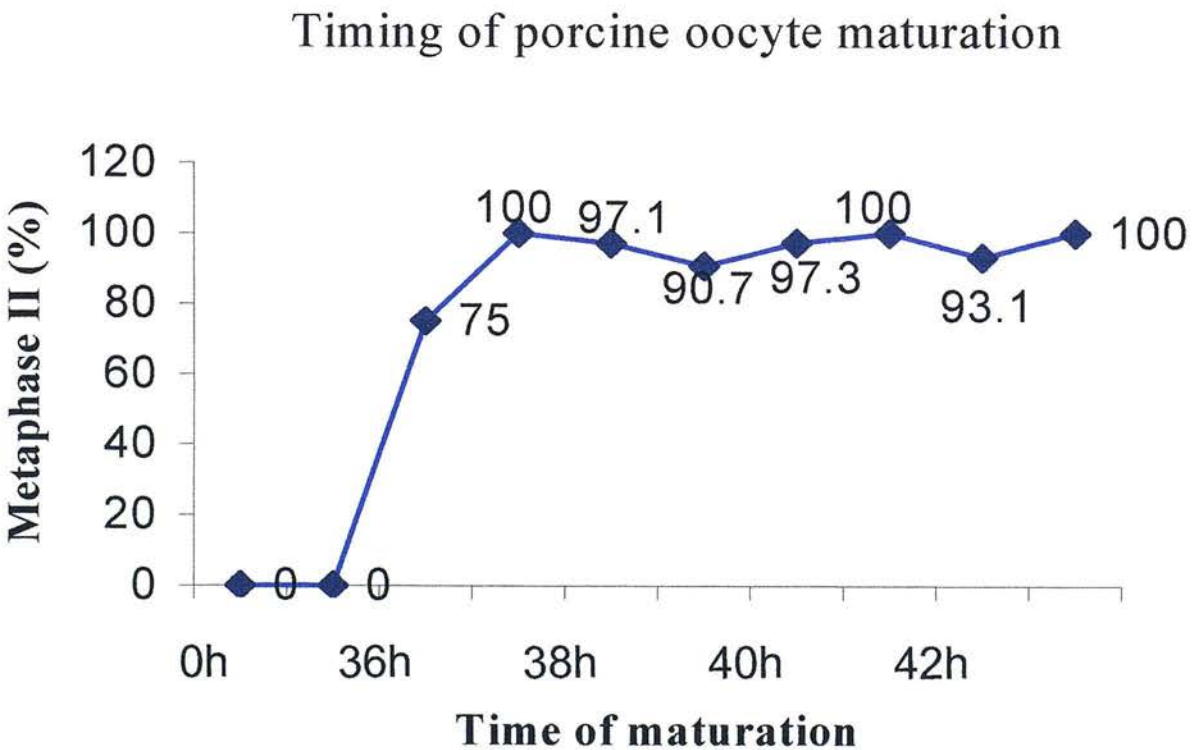

Timing of porcine oocyte maturation
Metaphase II (%)
120
100
80
60
40
20
0
0
0
75
100
97.1
90.7
97.3
100
93.1
100
0h
36h
38h
40h
42h
Time of maturation

**Fig. 3.2.** *Interaction of timing of oocyte maturation and field strengths.* The curves represent the changes of the mean blastocyst rates of oocytes activated by a single pulse of 1.0, 1.25 and 1.5 kV/cm DC respectively, at 36, 40, 44, and 48 h of maturation (4x3). The curves show that the best timing of oocytes to be activated is 44 h of maturation except the treatment of 1.0 kV/cm. The curve of 1.0kV/cm clearly shows that blastocyst rate increases with ageing of oocytes.

**Interaction between oocyte age**

**and field strengths**

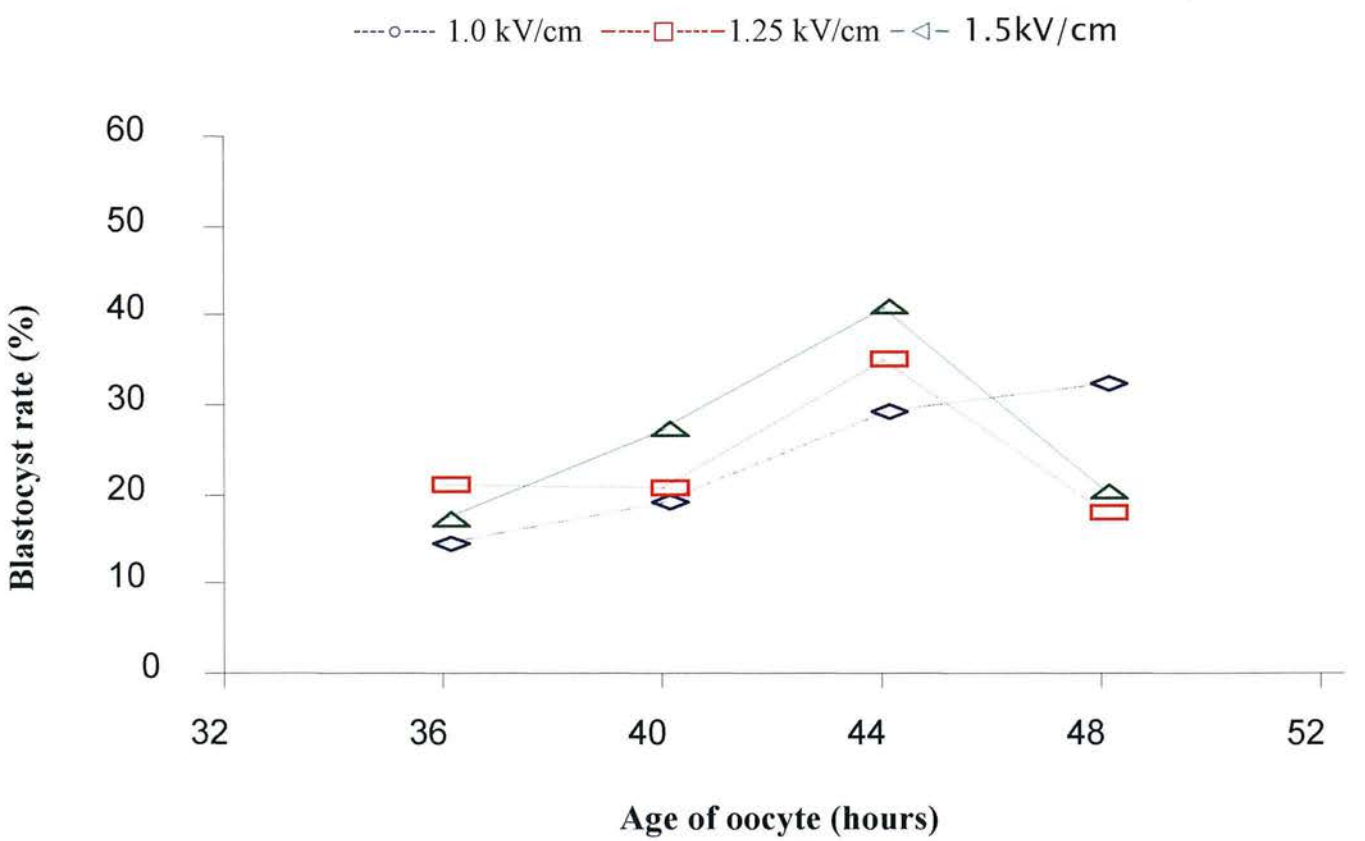


**Fig.3.3.** *Interaction of number of pulses and field strengths.* ** represents a significant difference across this bar and others ($p<0.01$ or 0.05) expect 40.5% ($p<0.05$). The bars represent the mean blastocyst rate (Means $\gamma$ SEM) of activated oocytes cultured *in vitro* for 7 days. 44 h-post-matured oocytes were activated by 1, 3, 5 x 80μsec.consecutive pulses of 1.0, 1.25 and 1.5 kV/cm DC (3x3), respectively. 3 x 80μsec. pulses of 1.0 kV/cm DC yielded the highest blastocyst rate of 54.9%, which was significantly higher than the others bars ($p<0.01$) except the bar representing 5x 80μsec.consecutive pulses of 1.0 kV/cm DC ($p<0.05$).

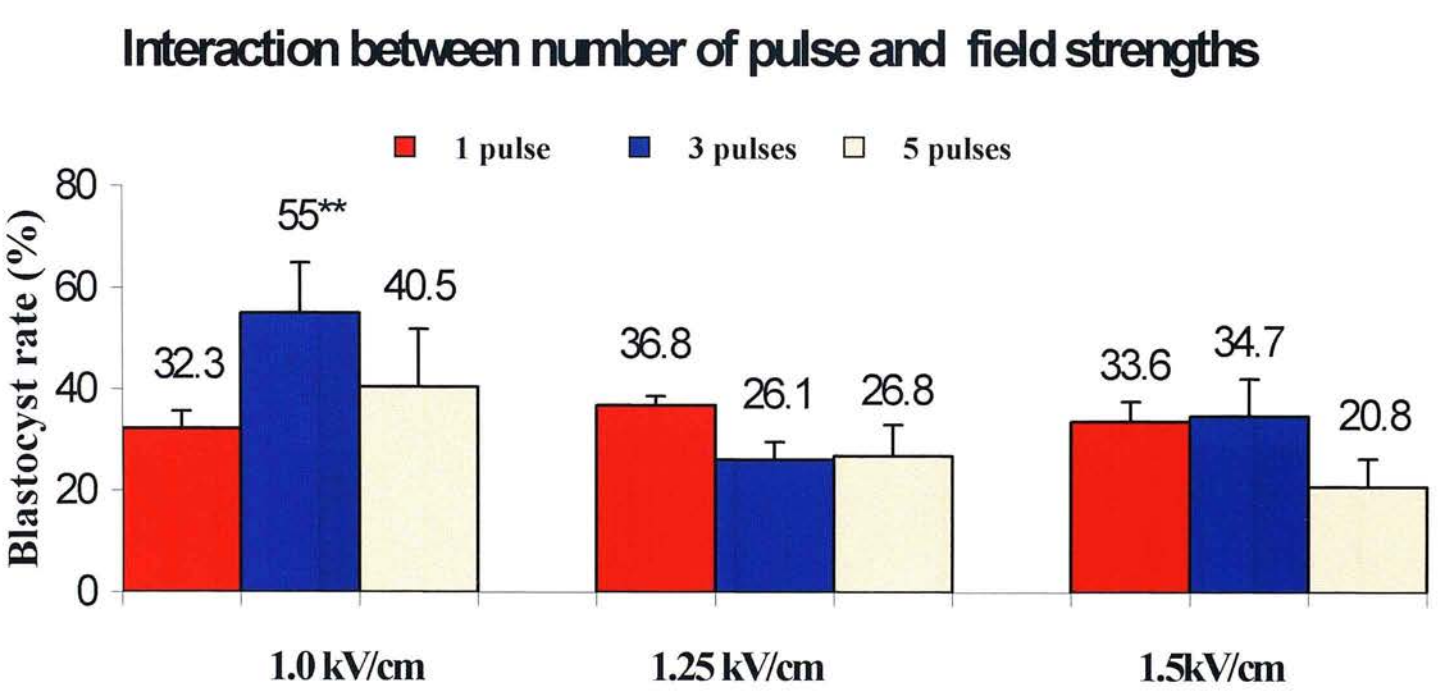

Interaction between number of pulse and field strengths
1 pulse
3 pulses
5 pulses
Blastocyst rate (%)
80
60
40
20
0
32.3
55**
40.5
36.8
26.1
26.8
33.6
34.7
20.8
1.0 kV/cm
1.25 kV/cm
1.5kV/cm

**Fig.3.4.** *Interaction of number of pulses and pulse duration.* Oocytes were activated by 1 or 3 consecutive pulses of 1.0 kV/cm for 20, 40, 60, 80, 100, 120, 140 and 160 μsec. respectively (2x8). The curves represent the mean blastocyst rates of oocytes activated by single or 3 consecutive pulses. Each of the treatments contained at least 230 oocytes in 7 replicates. The mean blastocyst rates for 60 and 80 μsec.with 3 consecutive pulses were significantly higher than those with a single pulse ($p<0.05$), but not different from that for 100μsec. with a single pulse ($p>0.05$).

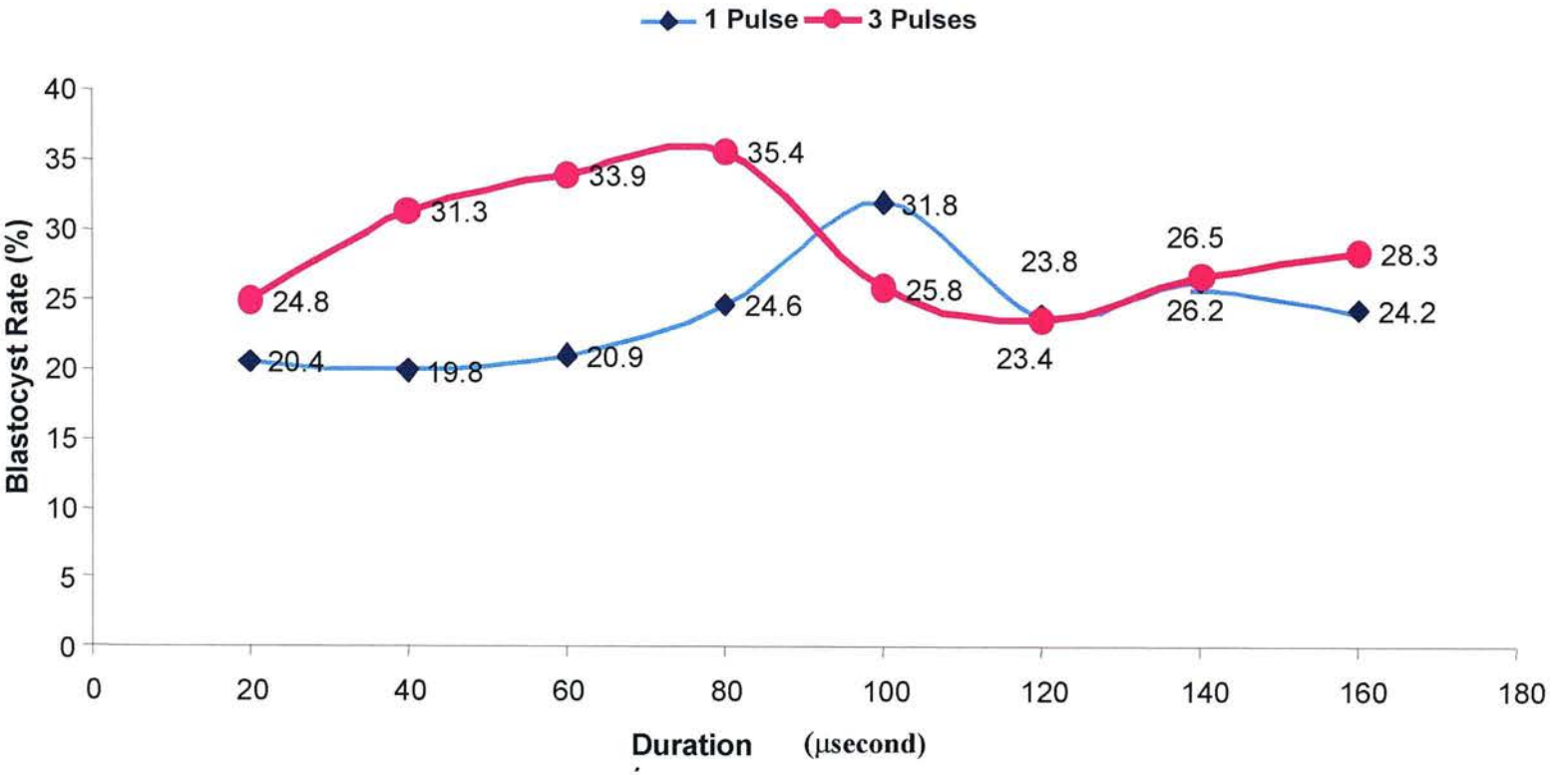

Interaction of number of pulses and field strengths
1 Pulse
3 Pulses
Blastocyst Rate (%)
40
35
30
25
20
15
10
5
0
0
20
40
60
80
100
120
140
160
180
Duration (μsecond)
24.8
31.3
33.9
35.4
25.8
23.4
26.2
28.3
20.4
19.8
20.9
24.6
31.8
23.8
26.5
24.2

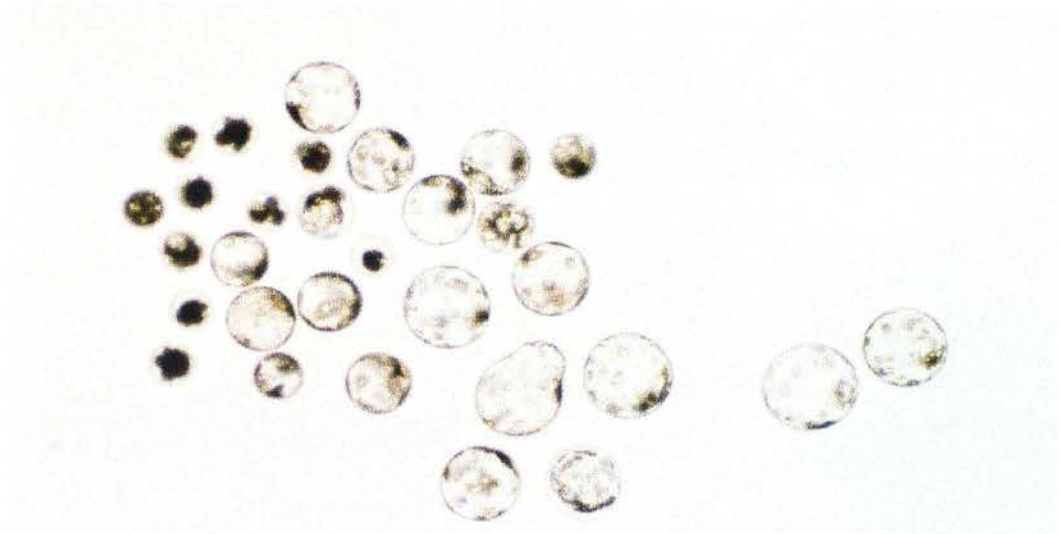
**Table 3.2. A comparison of karyotypes of IVM parthenogenetic and *in vivo* fertilised blastocysts**

| Type of blastocysts | No. of karyotyped (N) | No. of spread* (N) | No. of cells with spread chromosomes **(N) | No. of 2N blastocysts (N) | No, of 4N blastocysts (N) | No. of mixed ploidy (N) | Percentage of 2N blastocysts (%) |
|---|---|---|---|---|---|---|---|
| *In vivo* fertilised | 21 | 9 | 1-4 | 9 | 0 | 0 | 100 |
| IVM parthenogenetic | 130 | 73 | 1-9 | 61 | 7 | 5 | 83.6 |

No significant difference in percentage of diploid blastocysts between the two groups ($p>0.05$; Chi square-Test).
Blastocysts with spread chromosomes; ** No. of cells with spread chromosomes in individual blastocysts

**Fig 3.5.** Parthenogenetic blastocysts that were produced by 3x 80 μsec.consecutive pulses of 1.0kV/cm following 0.25 kV/cm for 5 sec. and cultured *in vitro* for 7 days. (Magnifications: x 200)

**Fig. 3.6**. Nuclei of day 7 parthenogenetic blastocysts produced by the improved electrical activation protocol (Magnifications: x 400)



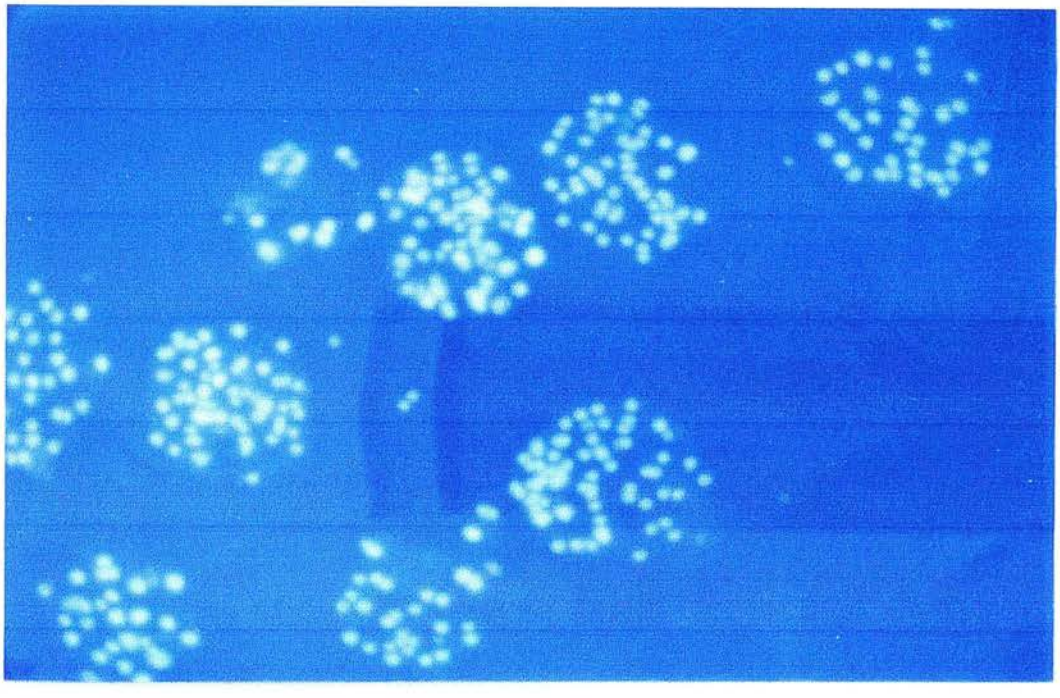
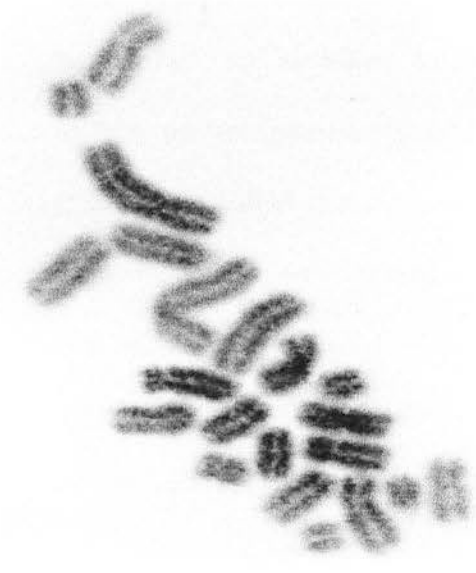


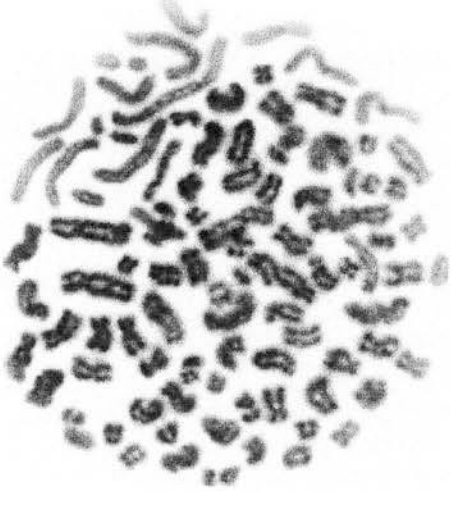
**Fig. 3.7. Karyotypes of parthenogenetic pig blastocysts.**
A haploid cell of a parthenogenetic pig blastocyst (**A**); Two diploid cells from two parthenogenetic pig blastocysts (**B, C**). A tetraploid cell of a parthenogenetic pig blastocyst (**D**);



A

B

C



D

## 3.6. Discussion

In these present studies, the interactions of various parameters influencing electrical activation of IVM pig oocytes were evaluated. The addition of amino acids but not EGF was beneficial for the developmental competence of activated oocytes to form parthenogenetic blastocysts. Pig oocyte activation was affected by oocyte age, electrical field strength, pulse number and pulse duration. There was evidence of interactions between oocyte age and applied voltage field strength, pulse number and pulse duration. Using an optimised protocol in the current study, the results show a high rate of *in vitro* development to the blastocyst stage with more than 80% having a normal karyotype (2n=38).

### 3.6.1. Effects of EGF and amino acids in maturation medium

EGF is usually added in maturation medium for pig oocytes in order to improve either nuclear or cytoplasmic maturation (Ding and Foxcorft. 1994; Illera, *et al.* 1998; Grupen *et al* 1997; Abeydeera *et al*, 1998). Based on these experiments the results show that oocytes matured in the presence of EGF in maturation medium can significantly decrease the incidence of polyspermy without a reduction of blastocyst rate after *in vitro* fertilisation (IVF) and that EGF can influence the developmental competence of oocytes (Abeydeera *et al*, 1998). Likewise, addition of amino acids including essential and non-essential amino acids in maturation medium also provides a benefit to promote nuclear maturation and male nuclear formation, which is normally regarded as an important indicator of cytoplasmic maturation following IVF (Ka *et al.* 1997). However, McGaughey (1985) suggested that amino acids could induce non-disjunction during *in vitro* maturation of porcine

oocytes. Non-disjunction of chromosomes could result in production of oocytes with aberrant chromosome numbers. He also concluded that the amino acid influence was not only dose dependent for porcine oocytes but also was species specific. In addition, it has been well known that either EGF or amino acids indirectly influence oocytes through gap junction channels between their cumulus cells and the oocyte (Mattioli *et al* 1988). The data presented in Table 3.1 obviously indicated that addition of amino acids in maturation medium promoted development of parthenogenetically activated porcine oocytes to the blastocyst stage although the blastocyst rate was not significantly different from that in the control ($p>0.05$), whereas adding EGF in maturation medium seemed not to have any positive effect under the conditions. There was no significant difference in maturation rate among the treatments (not shown data). The blastocyst rate in EGF alone or EGF combined with AA was significantly lower than that in AA alone ($p<0.05$). Furthermore, when adding AA into the maturation medium containing EGF, the blastocyst rate appeared to increase from 20.2 to 25.6%, which was in correspondence to the result showing a positive effect of AA in the medium containing AA alone. Although the detailed mechanism(s) of parthenogenetic activation and fertilisation has not been well known, some reports (Wang *et al*, 1998; Jung. *et al* 1998) have suggested that blastocyst metabolism from fertilisation and parthenogenetic activation is different and that activation pathway(s) between fertilisation and parthenogenetic activation might differ or overlay. If that hypothesis would be true, one possible explanation to the different effect of EGF and AA may be that oocytes would require "maturation" of different pathway(s), which could differently be enhanced by adding EGF or AA for fertilisation or parthenogenetic activation. Perhaps, there may be an alternative

explanation that MII oocytes matured in the presence of either EGF or AA in maturation medium may need to be effectively activated by different stimuli such as voltages, number of pulses and pulse duration since only 3 x 80 μsec consecutive pulses of 1.25 kV/cm DC was used for all the treatments in this experiment. In Fig 3.1, maturation rates of oocytes matured in NCSU23 medium supplemented with 10% pFF, 0.6 mM cysteine and AA at different time points of maturation were similar to the results reported by others (Wang *et al*, 1994; Ka *et al.* 1997) although different maturation media were used. From this point, nuclear maturation in pig oocytes seems not to be highly influenced by adding AA in maturation medium, but cytoplasmic maturation may be improved.

In general, although the mechanism(s) of how EGF and AA in maturation medium can influence developmental competence of parthenogenetically activated porcine oocytes remains unknown, these results imply that EGF and AA in maturation medium may influence porcine oocyte activation differently. Under these conditions, addition of amino acids in maturation medium appeared to have a positive effect on developmental competence of activated oocytes, whereas EGF did not. However, the incidence of non-disjunction of porcine chromosomes, which may be induced by adding amino acids in maturation medium, needs to be further investigated.

### 3.6.2. Effect of oocyte age and field strength

In Fig 3.2, the curves suggest that developmental competence of electrically activated oocytes depends on age of oocytes and peaks at 44 h of maturation, 7 h after over 90% of IVM oocytes had arrested at the MII stage (Fig 3.1). Interestingly,

this time of 44 h of maturation is also the best time of maturation for *in vitro* fertilisation of pig oocyte in the same *in vitro* maturation system of pig oocytes (Funahashi et al., 1994), which indicates that the timing of pig oocyte maturation required for either parthenogenetic activation or *in vitro* fertilisation is same or similar. Age of the oocyte is the single most important factor influencing oocyte activation. In contrast to fusion rate, activation rate increases with ageing of the oocyte (Robl *et al*, 1992). This indicates that developmental competence of activated oocytes may be more related to cytoplasmic maturation rather then nuclear maturation. It has been well known that during fertilisation, sperm can trigger $Ca^{2+}$ oscillations in oocyte, which are critical to initiation of development following either parthenogenetic activation or fertilisation (Carroll *et al* 1996). In addition, there is evidence showing that during maturation changes to the inherent properties of $Ca^{2+}$ channels, the size of the $Ca^{2}$ store and the density of the channels may all contribute to increase sensitivity of regenerative $Ca^{2}$ release (Carroll, *et al* 1998). Therefore, the difference between oocytes responding to the stimuli at different time point may reflect increases in the ability of oocyte cytoplasm to respond to electrical stimuli during the final period of maturation. Kikuchi *et al* (1995) observed that the gradual decrease of histone H 1 kinase activity was correlated with ageing of oocytes matured *in vitro* and with their ability to be parthenogenetically activated. This is consistent with the results showing lower electrical field strength being able to obtain more development from aged oocytes. Oocyte age dependent of oocyte activation has been observed in several species such as bovine (Collas *et al*; 1992; Ware *et al,* 1989), mouse (Collas *et al*, 1989) and pigs (Hagen *et al*, 1991; Prochazka, *et al;* 1992). However, most of these observations were based on

activation rate, namely pronuclear formation rather than development to the blastocyst stage. In the cow, developmental competence has been observed in aged oocytes (Collas *et al* 1992). The current results also suggest that age of oocyte influence not only activation rate but also developmental competence of activated oocytes in pigs. More importantly, in this experiment, an optimum period to activate IVM pig oocytes has been confined to a "narrower window " than in other species (Fig 3. 2), which is less than 8 h.

### 3.6.3. Interaction of pulse number and field strength

In this experiment, the results indicated that better development could be obtained using multiple pulses only when combined with lower field strength. This observation is similar to previous reported in bovine oocyte activation (Collas *et al,* 1993). During normal fertilisation, sperm triggers $Ca^{2+}$ oscillations in oocytes, these $Ca^{2+}$ oscillations result in a sequence of events such as exocytosis of the cortical granules, which prevents the penetration of supernumerary spermatozoa, the completion of meiosis and entry into the first embryonic mitosis although there are differences between species in the number and duration of the oscillations (Carroll *et al* 1996).. Therefore, multiple pulses to activate oocytes presumably mimic the $Ca^{2+}$ oscillations of fertilised oocytes more closely than a single pulse. Multiple pulses have shown to have a benefit to oocyte activation and the developmental competence of activated oocytes in several species such as mouse (Vitullo *et al* 1992), rabbits (Collas *et al* 1990; Ozil, 1990), cattle (Collas *et al*, 1993) and pigs (Grupen *et al*, 1999). However, in those studies, the given pulses were spaced at intervals of several min up to 30 min in order to mimic the $Ca^{2+}$ oscillations that

occur during fertilisation. Perhaps, the mechanism of these multiple pulses with no intervals between the given pulses is likely to be different. It seems to be impossible that such multiple pulses could trigger the $Ca^{2+}$ oscillations that occur at fertilisation or are mimicked by the multiple pulses with intervals of several min. However, the combination of multiple pulses with low field strength for a short duration may cause only a single speak of $Ca^{2+}$ whose shape and duration may differ from a single pulse, resulting in less damage of the plasma membrane of activated oocytes compared with a single pulse of higher field strength for a longer duration. Recently, it has been reported that a single pulse of higher electric field strengths for a longer duration is capable of producing normal parthenote fetuses up to day 30 in cattle (Fukui *et al* 1992), day 24 in sheep (Loi *et al*, 1998) and 29 days of gestation in pigs (Kure-bayashi *et al*, 2000). However, in the latter study, the rate of degenerated oocytes was higher (4.6-15%) than that was observed with this protocol (very few oocytes degenerated after activation). In Fig 3. 3, it is clearly shown that the highest mean blastocyst rate was yielded by 3 x 80 μsec consecutive pulses of 1.0 kV/cm DC. Also, these results indicate that multiple pulses of higher electrical field strength or too many pulses seem to be not beneficial for the developmental competence of activated oocytes since the oocytes could be damaged by the over-stimulation.

### 3.6.4. Interaction of pulse number and pulse duration

Pulse duration seems to be not important compared with field strength and pulse duration (Robl *et al* 1992). Here, I describe an interaction between pulse number and pulse duration in pig oocyte activation. In Fig 4, the curves show that

the best development is achieved with 3 pulses and a broad range of duration (40-80 μsec), whereas a single pulse requires a longer duration (100 μsec) to approach this result. Although high quality oocyte were not strictly selected in this experiment because a large number of oocytes were required and the blastocyst rates were generally lower, the best blastocyst rate observed in the treatment of 3 consecutive pulses was still yielded by the same stimuli as that in Fig 3. 3. Therefore, this demonstrates that this protocol is not only efficient but also repeatable. These results support the observation that optimised pulse duration with 2 pulses of 1.2 kV/cm DC was 30–60 μsec which was used to fuse and activate reconstructed pig embryos by transferring embryonic cells to enucleated oocytes, resulting in the births of cloned piglets (Prather *et al*, 1989). In the present experiment, the results demonstrate that 3 consecutive pulses can not only improve the developmental competence of activated oocytes but also create a "wider window" for activating pig oocytes compared with a single pulse.

In this chapter, I established an in vitro maturation system for porcine oocyte activation and investigated the interactions across oocyte age, field strength, and numbers of pulses and pulsing duration, subsequently optimising the current electrical activation protocol for IVM porcine oocytes. Using the improved protocol has resulted in a high blastocyst rate and normal karyotype (2N) in over 80% blastocysts. Therefore, it is concluded that the effective electrical protocol of IVM porcine oocyte activation is 3 x 80 μsec consecutive pulses of 1.0 kV/cm DC following 0.25 kV/cm AC using 44 h-post-matured oocytes under the laboratory conditions. Furthermore, I observed that using 3 consecutive pulses of 1.0 kV/cm DC could create "a wider window" for oocytes activation compared to using a single

pulse of the same field strength. In addition, the activation procedure optimised in these studies may have two important roles during nuclear transfer. First, it provides an effective method for activation of reconstructed embryos (DeSousa *et al*, 2001, BOR accepted). Secondly, it might be used to produce parthenotes to maintain pregnancy after transfer of cloned embryos (King, *et al,* 2001, submitted).

# Chapter 4

# Effects of Activation Conditions: Temperature, Activation Media, Concentration of $Ca^{2+}$ and $Mg^{2+}$ in Activation Medium and Diploidisation

## 4.1. Introduction

In animal cloning, when using metaphase II (MII) oocytes as recipient oocytes, the method of activation is critical for subsequent development. In pigs, although current activation protocols including chemical and electrical activation can stimulate pronuclear formation, cleavage and development to the blastocyst stage, both the frequency of development and the quality of the embryos produced are low (Polejaeva *et al*, 2000). One activation protocol to activate reconstructed pig embryos injected donor cells from fetal fibroblasts into enucleated ovulated oocytes resulted in the birth of a cloned piglet, the same protocol was however used to activate *in vitro* matured (IVM) pig oocytes and yielded a blastocyst rate of only 2.4% (Onishi *et al,* 2000). Although chemical activation has been used in animal cloning such as in sheep (Loi *et al* 2000), cattle (Wells *et al.,* 1999) and pigs (Betthauser *et al.,* 2000), electrical activation is still widely used for nuclear transfer in cattle (Kato *et al*, 2000), sheep (Campbell *et al* 1996; Wilmut *et al* 1997) and pigs (Polejaeva *et al*, 2000; Onishi *et al*, 2000; Li *et al*, 2000). Many factors influencing the efficiency of pig oocyte electrical activation have been investigated by different

laboratories (Liu and Moor, 1997; Robl *et al* 1992; Wang *et al* 1998; Collas P and Robl J M. 1990; Kono *et al* 1989; Aoyai, 1992). To date, it has been reported that pig oocyte electrical activation can be affected by many factors such as quality of oocytes including oocyte age (Ware *et al* 1989; Kikuchi *et al*; 1995; Collas *et al* 1989), field strength (Collas P and Robl J M; 1990; Collas *et al* 1993), pulse number (Collas *et al* 1993; Robl *et al*, 1992; Liu and Moor, 1997), pulse duration (Robl *et al*, 1992), $Ca^{2+}$ and $Mg^{2+}$ concentration in activation medium (Robl *et al*, 1992), karyotype of activated oocytes (Kim *et al.*, 1997; Kure-bayashi *et al.*, 1996) and type of activation medium (Robl *et al.*, 1992; Liu and Moor, 1997). In addition, the $Ca^{2+}$ transient caused by electrical stimulation is the result of an influx of calcium; it would then be expected that calcium would be required in activation medium (Robl *et al.*, 1992; Ozil, 1990). Ozil (1990) has verified that $Ca^{2+}$ is essential for activating rabbit oocytes. Also, no spike of calcium in pig oocytes in activation medium containing no calcium was observed after electrical stimulation (Sun *et al.*, 1992). However, the calcium peak seems not to be related to activation, concentration of calcium in activation medium may not be critical for activation (Robl *et al* 1992). For instance, in mouse and pig oocytes, a marked increase of $Ca^{2+}$ occurs even in the absence of extracellular $Ca^{2+}$, indicating that part of the $Ca^{2+}$ originates from intracellular stores (Carroll *et al*, 1996). It is relevant that $Ca^{2+}$ plays an important role in pig oocyte activation although some results are conflicting. In the last chapter, oocyte age and electrical parameters have been optimised and an improved activation protocol has basically been determined. In this chapter, I focused on investigating the effect of activation conditions including temperature, type of

activation medium, $Ca^{2+}$ and $Mg^{2+}$ concentrations in activation medium and diploidization of activated oocytes on development of activated oocytes.

In this chapter, three studies with a total of 6 experiments were carried out. In the first study, a comparison of activation temperature being 25° and 37°C were made and three activation media including Mannitol or Sorbitol, 0.1 mM $Mg^{2+}$, 0.05 mM $Ca^{2+}$ and Zimmermann medium were compared; in the second study, Mannitol alone, Mannitol with either $Ca^{2+}$ or $Mg^{2+}$; Mannitol with different concentrations of either $Ca^{2+}$ or $Mg^{2+}$ were examined. In the last study, activated oocytes were treated in cytochalasin B (CB) for 0-6 h in order to determine a minimum duration of CB treatment for development of activated pig oocytes. Some blastocysts in each group were karyotyped meanwhile some activated oocytes were fixed 12–16 h after CB treatments to determine statues of pronuclear formation. In addition, CB and CB combined with cycloheximide (CH) after activation of IVM pig oocytes were compared.

The results from the three studies show that temperature at activation and $Ca^{2+}$ concentration in activation medium are important factors influencing developmental competence of the activated pig oocytes, whereas $Mg^{2+}$ in activation medium seems not to be essential for pig oocyte activation. Moreover, a minimum duration of CB treatment seemed to require 3 h, which resulted in a mean blastocyst rate of 39.8% with 73.3% of the blastocysts being diploid, both the mean blastocyst rate and the proportion of the diploid blastocysts were significantly higher than those of the oocytes treated without CB ($P<0.01$).

## 4.2. Materials and Methods

### 4.2.1. Collection of cumulus oocyte complexes (COCs) and *in vitro* maturation

Oocyte collection and *in vitro* maturation were based on the protocol described by Abeydeera *et al* (1998). Briefly, pig ovaries were collected from a local slaughterhouse. Ovaries were transported to the laboratory at 25°-30°C, then rinsed three times in a plastic beaker with warmed PBS and kept in a water bath at 30°C. COCs were aspirated from ovarian follicles 3-8 mm in diameter. Subsequently, oocytes with at least three layers of cumulus cells were selected and rinsed three times in Hepes–buffered–TL-PVA and three times in maturation medium comprising BSA-free NCSU23 medium, 10% pig follicular fluid (pFF), 0.6 mM cysteine, 1% essential amino acids and 0.5% non-essential amino acids (v/v). Groups of 50 COCs were cultured in 500 µl drops of maturation medium supplemented with 10 IU/ml eCG (Intervet, UK Ltd, Cambridge UK) and 10 IU/ml hCG (Intervet, UK Ltd, Cambridge UK) for 22 h, at 39°C, 5% $CO_2$ in air. 22 h later, COCs were rinsed three times in fresh maturation medium without hormonal supplements, finally cultured in hormones-free maturation medium for an additional 22 h period under the same conditions.

### 4.2.2. Oocyte activation by electrical stimulation and embryo culture

Post 44 h matured COCs were placed on a warm stage at 37°C and their cumulus cells were removed by repeated pippetting. Denuded oocytes were rinsed twice in $Ca^{2+}$-free–Hepes-buffered NCSU 23 medium and twice in activation medium containing 0.3 M Mannitol, 0.05 mM $Ca^{2+}$ and 0.1 mM $Mg^{2+}$. Oocytes were

transferred to a 200 μl drop of the activation medium in a 200 μm gap between two parallel electrodes in a glass chamber that was on a warm stage at 37°C and activated by 3 x 80 μsec consecutive pulses of 1.0 kV/cm DC following 0.25 kV/cm AC for 5 sec. Activated oocytes were transferred into 50 μl drops of 7.5 μg/ml cytochalasin B in NCSU 23 medium with 0.4% BSA (A-6003, Sigma) and cultured for 6 h, at 39°C, 5% $CO_2$ in air. After CB treatment, groups of 30-40 activated oocytes were cultured in 500 μl drops of NCSU 23 medium with 0.4% BSA for 7 days at 39°C, 5% $CO_2$ in air.

### 4.2.3. Nuclear staining of porcine oocytes and staining of nuclei in parthenogenetic blastocysts

Activated oocytes cultured in NCSU23 medium with 0.4% BSA 12–16 h after CB treatment from 0 to 6 h were fixed and stained to assess pronuclear formation. They were washed several times in Hepes–buffered NCSU 23 medium containing 0.4% BSA. Groups of 10-30 oocytes were fixed in acetic acid : methanol (1:3) for a minimum of 48 h, and stained with 1% orcein (O-7380, Sigma).

Nuclear count of blastocysts was performed by staining blastocysts with Hoechst 33342. Briefly, day 6 or day 7 blastocysts were transferred into 50 μl droplets of the same medium containing 5 μg/ml Hoechst 33342 and cultured in the incubator for 10–15 min. After incubation, the blastocysts were gently mounted on a cleaned glass slide and covered with a cleaned glass coverslip. The slides were observed under UV light.

### 4.2.4. Experimental designs

#### 4.2.4.1. Study 1, Effects of temperature and activation medium at electrical activation

##### 4.2.4.1.1. *Experiments 1: Effect of temperature at activation*

44 h–post-matured oocytes were randomly divided into two groups. Oocytes in group 1 were placed on a warm stage controlled by a temperature regulator except which, an indicator on the stage also digitally displaying temperature on the surface of the stage. In-group 2, oocytes were handled at room temperature (25°C) controlled by an air conditioner. In both groups, cumulus cells removing and oocyte activation were performed at the temperature of either 25 or 37°C within 30 min. After activation, all oocytes were treated in CB for 6 h, then cultured for 7 days at 39°C, 5% $CO_2$ in air. The experiment was repeated 5 times with a total of at least 340 oocytes in each treatment.

##### 4.2.4.1.2. *Experiment 2: Effect of activation medium*

This experiment was to compare three common activation media. 44 h-post–matured oocytes were washed three times with $Ca^{2+}$ free–Hepes buffered NCSU 23 medium and three times in activation medium required and activated by 0.25 kV/cm AC for 5 sec followed 3x 80 μsec consecutive pulses of 1.25 kV/cm DC in (1) 0.3 M Mannitol, 0.05 mM $Ca^{2+}$, 0.1 mM $Mg^{2+}$ (M); (2) 0.3 M Sorbitol, 0.05 mM $Ca^{2+}$, 0.1 mM $Mg^{2+}$ (S) and (3) Zimmermann medium containing 0.5mM $Mg^{2+}$, 0.1 mM $Ca^{2+}$

(Z), respectively. All oocytes were treated in CB for 6 h, then cultured for 7 days at 39°C, 5% $CO_2$ in air. The experiment was conducted in five replicates, and each of three treatments contained a total of 357-381 oocytes.

### 4.2.4.2. Study 2. Effects of $Ca^{2+}$ and $Mg^{2+}$ in activation medium.

#### 4.2.4.2.1.. *Experiment 3. Effect of $Ca^{2+}$ in activation medium*

The aim of this experiment was to investigate effect of $Ca^{2+}$ in activation medium on development of activated oocytes to obtain the adequate concentration of $Ca^{2+}$ in activation medium for IVM porcine oocytes. 44 h post matured oocytes were electrically activated in (1) 0.3 M Mannitol alone (M), (2) 0.3 M Mannitol, 0.1 mM $Mg^{2+}$ (MM), (3) 0.3 M Mannitol, 0.1 mM $Mg^{2+}$, 0.05 mM $Ca^{2+}$ (MMCL), (4) 0.3 M Mannitol, 0.1 mM $Mg^{2+}$, 0.1 mM $Ca^{2+}$ (MMCH), respectively. In order to control the required concentrations of both $Ca^{2+}$ and $Mg^{2+}$ in the activation media all oocytes were cultured in $Ca^{2+}$ free and $Mg^{2+}$ free NCSU 23 medium for 15 min, then washed three times in $Ca^{2+}$ and $Mg^{2+}$ free-Hepes buffered NCSU 23 medium and three times in the activation medium required before electrical activation Also, all the media and glass pipettes used for transferring oocytes needed to be changed after having been used once. All the activated oocytes were immediately transferred into the culture medium containing 7.5μg/ml CB and cultured for 6 h, they were then cultured *in vitro* for 7 days. This experiment was repeated four times with a total number of 258-277 oocytes in each treatment.

#### 4.2.4.2.2. *Experiment 4. Effect of $Mg^{2+}$ in activation medium*

This experiment was designed to examine the effect of $Mg^{2+}$ in activation medium. This experiment was carried out in the same way as those in experiment 3. Briefly, 44-h post-matured oocytes were washed three times in $Ca^{2+}$ and $Mg^{2+}$ free-Hepes buffered NCSU 23 medium but not cultured in $Mg^{2+}$ and $Ca^{2+}$ free NCSU 23 medium for 15 min prior to activation, and washed three times in the activation medium required. They were then electrically activated in (1) 0.3 M Mannitol alone (M), (2) 0.3 M Mannitol, 0.05 mM $Ca^{2+}$ (MCL), (3) 0.3 M Mannitol, 0.05 mM $Ca^{2+}$, 0.05 mM $Mg^{2+}$ (MCML), (4) 0.3 M Mannitol, 0.05 mM $Ca^{2+}$, 0.1 mM $Mg^{2+}$ (MCMH), respectively. Afterwards, all the oocytes were immediately transferred into the culture medium containing CB and incubated for 6 h; they were then cultured for 7 days. Each of these four treatments contained a total number of 236 to 264 oocytes in 4 replicates

### 4.2.4.3. Study 3. Effect of diploidisation of activated oocytes

#### 4.2.4.3.1. *Experiment 5 Effect of CB treatment*

This experiment was designed to investigate effect of CB treatment and karyotype on developmental competence of porcine oocytes after electrical activation. 44-h post-matured oocytes were electrically activated in 0.3 M Mannitol, 0.1 mM $Mg^{2+}$, 0.05 mM $Ca^{2+}$, and then they were randomly divided into 6 groups and treated in CB for 0, 1, 2, 3, 4, and 6 h. After CB treatment, all activated oocytes were cultured for 7 days. Some blastocysts were karyotyped in order to determine their karyotypes. In addition, some oocytes of these groups were fixed 12-16 h after

activation to examine pronuclear formation. Each of treatments contained 273–337 oocytes in 7 replicates.

#### 4.2.4.3.2. *Experiment 6. A comparison of CB and cycloheximide (CH)*

This experiment was conducted to examine effect of CB and CH treatments on karyotype of parthenogenetic blastocysts. 44-h post-matured oocytes were electrically activated. Activated oocytes were then randomly divided into three groups, which were treated with either CB alone, or 7.5 μg /ml CB combined with 10 μg / ml CH, or 10 μg/ml CH alone, respectively. Oocytes treated with CB alone for 6 h were used as a control, and the other two groups were treated for 4 h. After diploidization, oocytes were cultured *in vitro* for 7 days. Also, some blastocysts from the groups were karyotyped, the rest were stained with Hoechst 33342 for nuclear count. This experiment was performed in 7 replicates with a total of 404-436 oocytes in each treatment.

### 4.2.5. Statistical analysis of the data

All data collected were analysed by Excel, Student's T-test or $\chi^2$-test. Probability of 0.05 was regarded to be significant difference.

## 4.3. Results

### 4.3.1. *Effect of activation temperature*

In experiment 1, the mean blastocyst rate of 43.5% (149/341 oocytes) in oocytes activated at 37°C was significantly higher than 32.0% (120/371) in oocytes

activated at 25°C ($p<0.01$;), which showed that the mean blastocyst rate of activated oocytes was highly related to temperature change. However, there was no difference in the mean number of nuclei / blastocyst ($P>0.05$) (Table 4.1).

### 4.3.2. *Effect of activation medium*

In experiment 2, the mean blastocyst rates of M, S and Z were 35.5% (135/374), 37.8% (135/381) and 40.1% (145/357), respectively. No significant differences among the three treatments in the mean blastocyst rate were observed ($p>0.05$). In addition, the mean number of nuclei/blastocyst in M, S and Z was 45.39±15.88, 51.70±20.72 and 46.12±21.14, respectively. There were no significant differences across the treatments ($p<0.05$) (Table 4. 2). Also, approximate 10% of the activated oocytes were able to develop to hatching or hatched blastocysts (Fig 4. 3).

### 4.3.3. *Effect of $Ca^{2+}$ in activation medium*

In experiment 3, the mean blastocyst rates of oocytes activated in M, MM, MMCL and MMCH were 24.4% (65/259), 16.1% (45/270), 44.0% (116/258) and 26.0% (78/277), respectively. Of these, MMCL yielded the highest mean blastocyst rate of 44.0% that was significantly higher than other three treatments ($p<0.05$). However, no differences in the mean blastocyst rate among M, MM and MMCH were observed ($p>0.05$) (Fig 4 1). Also, no differences in the mean number of nuclei/blastocyst among the treatments were found ($p>0.05$; data not shown)

### 4.3.4. *Effect of $Mg^{2+}$ in activation medium*

In experiment 4, the results show that MC yielded the highest mean blastocyst rate of 47% (125/264), which was not significantly different from those of MCML (37.9%, 94/256) and MCMH (42.5% 103/248) ($p>0.05$), but significantly higher than 34.4% (71/207) of M ($p<0.01$). Although there was no significant difference in the mean blastocyst rate between MCML and MCMH ($p>0.05$), the mean blastocyst rate of MCMH was significantly higher than M ($p<0.01$), whereas MCML was not significantly different from M ($p>0.05$) (Fig 4 2). Likewise, no differences in the mean number of nuclei/blastocyst among the treatments were observed ($p>0.05$; data not shown).

### 4.3.5. *Effect of cytochalasin B treatment*

In experiment 5, the mean blastocyst rates were 21.1% (72/337), 23.7% (68/294), 31.4% (90/273), 39.8% (110/274), 41.9% (119/284), and 42.5% (120/283) for 0, 1, 2, 3, 4 and 6 h of CB treatment, respectively. The proportions of diploid blastocysts at 0, 1, 2, 3, 4 and 6 h of CB treatment were 40, 43.5, 46.7, 73.3, 76.2 and 84.2% (Table 4.4.). The correlation coefficient ($R_1$) between the mean blastocyst rates and the proportions of diploid blastocysts was 0.959 ($p<0.01$). In addition, the mean proportions of oocytes with one polar body and two pronuclei that were regarded as diploid (diploid oocytes/activated oocytes) were 26%, 45.7%, 51.9%, 57.6%, 78.2% and 97.2% for 0, 1, 2, 3, 4, and 6 h of CB treatment, respectively (Table 4.3 and Table 4.4). The correlation coefficients ($R_2$) between the mean proportions of diploid blastocysts and the mean proportions of diploid oocytes, and ($R_3$) between the mean blastocyst rates and the mean proportions of diploid oocytes were 0.902 ($p<0.05$) and 0.89 ($p<0.05$), respectively. Surprisingly, no

significant differences in the mean number of nuclei/blastocyst among the treatments were observed ($p>0.05$) (Table 4.3).

### 4.3.6. *Effect of cytochalasin B and cycloheximide*

In experiment 6, the mean blastocyst rates of activated oocytes treated with CB alone, CB and CH, CH alone were 45.7, 42.9 and 17.3%, respectively (Table 4.5), there was a significant difference either between CB alone and CH alone treatments, or between CB + CH and CH alone treatments ($p<0.01$). However, no significant difference was found between CB alone and CB + CH treatments ($p>0.05$). Also, no differences in the mean number of nuclei/blastocyst were observed among the treatments ($p>0.05$) (Table 4.5). In addition, karyotypes of blastocysts were showed in Table 4.6. The proportions of the diploid blastocysts that were treated with CB alone, CB + CH and CH alone were 83.6, 81.3 and 53.8%, respectively. There was no significant difference in percentage of diploid blastocysts either between CB and CB + CH treatments or between CB + CH and CH alone ($p>0.05$), but a significant difference between CB alone and CH alone treatments ($p<0.01$).

**Table 4.1. Effect of temperature at activation on the parthenogenetic development of *in vitro* matured porcine oocytes**

| Temperature At activation | No. of oocytes | No. of cleaved | No. of blastocysts | Mean blastocyst rate (%) Mean ± SE | Mean number of nuclei/blastocyst Mean ± SE (n) |
|---|---|---|---|---|---|
| 25°C | 371 | 251 | 120 | 32.0 ± 11.2 | 32.20 ± 9.47 (101) |
| 37°C | 341 | 289 | 149 | 43.5±11.2** | 35.33 ± 11.36(59) |

** There is a significant difference in the mean blastocyst rate between 25° and 37°C ($p<0.01$, T–test). The experiment was repeated 5 times.

**Table 4.2. *In vitro* development of pig oocytes activated in three activation media**

| Medium | No. of oocytes | No. of D 2 cleaved oocytes | No. of blastocysts on day 7 | Mean blastocyst rate Mean ± SE (%) | Mean number of nuclei/ blastocyst Mean ± SE |
|---|---|---|---|---|---|
| Mannitol* | 374 | 303 | 135 | 35.5 ± 4.6 | 45.4 ± 15.9 |
| Sorbitol** | 381 | 295 | 135 | 37.8 ± 8.4 | 51.7 ± 20.7 |
| Zimmermann | 357 | 295 | 145 | 40.1 ± 10.6 | 46.1 ± 21.1 |

* Mannitol medium contains 0.3 M Mannitol, 0.05 mM $Ca^{2+}$ and 0.1 mM $Mg^{2+}$;
** Sorbitol medium contains 0.3 M Sorbitol, 0.05 mM $Ca^{2+}$ and 0.1 mM $Mg^{2+}$;
The recipe of Zimmermann's medium containing, 0.5mM $Mg^{2+}$ and 0.1mM $Ca^{2+}$ is seen in Appendix-V.

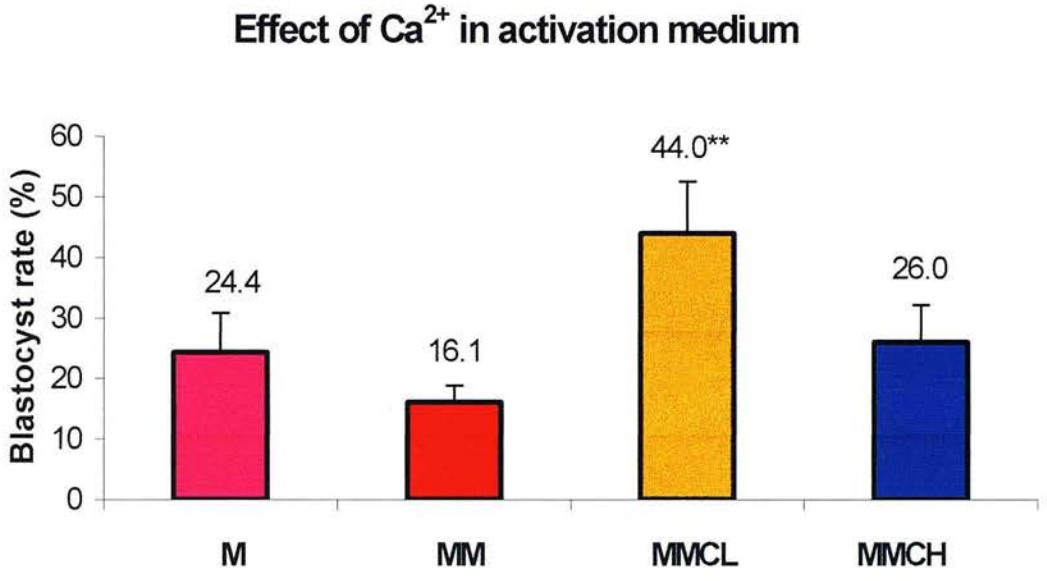
**Fig 4 1**. Effect of $Ca^{2+}$ concentration in activation medium on the blastocyst rate of electrically activated pig oocytes.

The bars represent the mean blastocyst rates with standard errors on day 7 of culture. The media M, MM, MMCL and MMCH represent 0.3M Mannitol alone, 0.3M Mannitol + 0.1mM $Mg^{2+}$, 0.3M Mannitol + 0.1mM $Mg^{2+}$ + 0.05 mM $Ca^{2+}$ and 0.3M Mannitol + 0.1mM $Mg^{2+}$ + 0.1 mM $Ca^{2+}$, respectively. The IVM oocytes were activated by 3 x 80 μsec consecutive pulses of 1.0 kV/cm DC following 0.25 kV/cm AC for 5 sec.

** shows that there is a significant difference between this treatment and others ($p< 0.05$ or 0.01, T-test).

**Fig 4.2**. Effect of $Mg^{2+}$ concentration in activation medium on the blastocyst rate of electrically activated pig oocytes.

The bars represent the mean blastocyst rates with standard errors on day 7 of culture. The media M, MC, MCML and MCMH represent 0.3M Mannitol alone, 0.3M Mannitol + 0.05mM $Ca^{2+}$, 0.3M Mannitol + 0.05 mM $Ca^{2+}$+ 0.05mM $Mg^{2+}$ and 0.3M Mannitol + 0.05 mM $Ca^{2+}$+ 0.1mM $Mg^{2+}$, respectively. The IVM oocytes were activated by 3 x 80 μsec consecutive pulses of 1.0 kV/cm DC following 0.25 kV/cm AC for 5 sec. The media MC and MCMH yielded significant higher mean blastocyst rates than that in medium M ($p<0.01$), but did not significantly differed from that in medium MCML ($p>0.05$). There was also no significant difference in the mean blastocyst rate between media M and MCML ($p>0.05$).


Effect of Ca2+ in activation medium
Blastocyst rate (%)
60
50
40
30
20
10
0
24.4
16.1
44.0**
26.0
M
MM
MMCL
MMCH

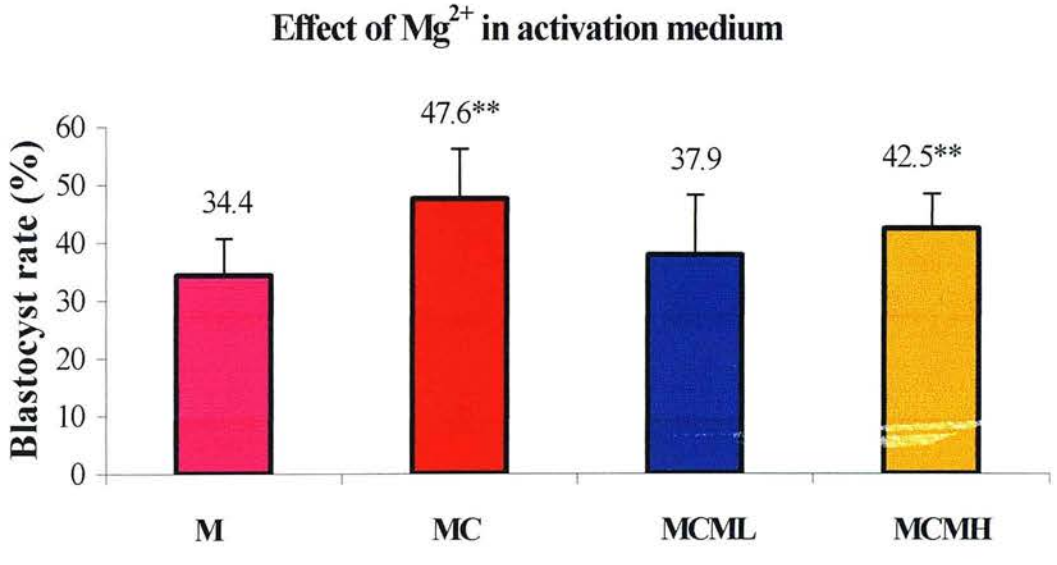

Effect of Mg2+ in activation medium
Blastocyst rate (%)
60
50
40
30
20
10
0
34.4
47.6**
37.9
42.5**
M
MC
MCML
MCMH

**Table 4.3. Effect of CB treatment**

| Item | 0 H | 1 H | 2 H | 3 H | 4 H | 6 H |
|---|---|---|---|---|---|---|
| **Pronuclear formation** | | | | | | |
| No. of examined oocytes | 77 | 83 | 83 | 71 | 81 | 90 |
| No. of activated oocytes | 74 | 71 | 74 | 61 | 74 | 80 |
| No. of 2N oocytes | 19 | 29 | 39 | 38 | 58 | 79 |
| Mean percentage of 2N (%) Mean±SD | 26±5.4 | 45.7±4.4 | 51.9±8.6 | 57.6±6.6 | 78.2±2.3 | 97.2±2.7 |
| **Blastocyst formation** | | | | | | |
| No. of oocytes | 337 | 294 | 273 | 274 | 284 | 283 |
| No. of D 2 cleaved | 260 | 227 | 199 | 211 | 225 | 219 |
| No. of D 7 blastocysts | 72 | 68 | 90 | 110 | 119 | 120 |
| Mean blastocyst rate (%) Mean ± SD | 21.1 ± 3.6[a] | 23.7 ± 6.1[a] | 31.4 ± 15.6 | 39.8 ± 13.0[b] | 41.9 ± 7.5[b] | 42.5 ± 9.2[b] |
| Mean number of nuclei / blastocyst Mean ± SD (n) | 33.5 ± 14.8 (82) | 30.6 ± 11.7 (89) | 30.8 ± 10.6 (78) | 31.9 ± 9.6 (90) | 33.5 ± 11.4 (92) | 31.6 ± 8.8 (108) |

The data on pronuclear formation are collected from 3 replicates and the data on blastocyst formation in 7 replicates.

Note: Mean% of 2N oocytes is based on no. of activated oocytes. There are significant differences in the mean proportion of 2N oocytes across the treatments ($p<0.05$ or 0.01, Student's T-test) except that between 2 and 3 h ($p=0.12$). The data were collected from three replicates.

There is significant difference between *a* and *b* ($p < 0.01$).

**Table 4.4. Effect of cytochalasin B treatment on karyotyped of parthenogenetic blastocysts**

| Item | 0 h | 1h | 2h | 3h | 4h | 6h |
|---|---|---|---|---|---|---|
| Number of blastocysts with spread chromosomes | 20 | 23 | 15 | 15 | 21 | 19 |
| Mean number of spread chromosomes per blastocyst | 3 (1-5) | 3 (1-5) | 2 (1-4) | 2 (1-4) | 1(1-2) | 3 (1-5) |
| 1N | 6 | 4 | 3 | 3 | 0 | 0 |
| 2N | 8 | 10 | 7 | 11 | 16 | 16 |
| 3N | 1 | 0 | 3 | 0 | 1 | 0 |
| 4N | 3 | 1 | 2 | 1 | 4 | 1 |
| Mixoploidy | 2 | 8 | 0 | 0 | 0 | 2 |
| 2N % | $40^{a}$ | $43.5^{a}$ | $46.7^{ac}$ | $73.3^{bc}$ | $76.2^{b}$ | $84.2^{b}$ |

There is a significant difference between *a* and *b* ($p<0.05$, $\chi^2$- Test).
1N =haploid, 2N = diploid, 3N = triploid, 4N = tetraploid.

**Table 4.5. Comparisons of CB and CH treatments**

| Item | No. of oocyte | No. of d2*cleaved oocytes | No of d 7* blastocysts (N) | No of d 7* blastocysts Mean ± SE(%) | Mean number of nuclei/blastocyst Mean ±. SE (N) |
|---|---|---|---|---|---|
| Cytochalasin B (CB) | 404 | 345 | 172 | 45.7 ± 11** | 41.9 ± 15.8 (114) |
| CB+ cycloheximide (CH) | 436 | 363 | 184 | 42.9 ± 12.9** | 38.1 ± 12.9 (137) |
| Cycloheximide | 428 | 340 | 71 | 17.3 ± 8.2 | 41.8 ± 20.5 (31) |

* d 2 and d 7 : day 2 and day 7. The data presented were collected in 7 replicates.

** There were significant differences between CB and CH and between CB + CH and CH ($p<0.0\ 1$).

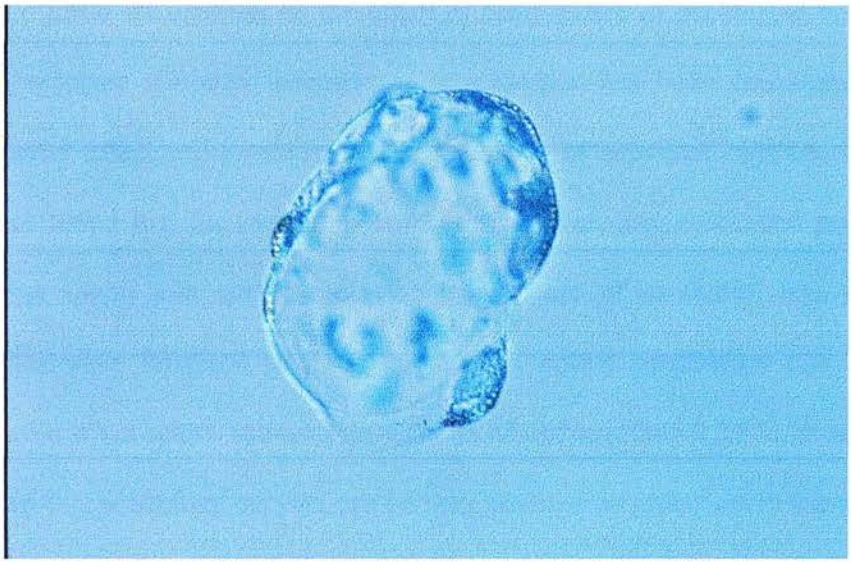
**Table 4.6. Effect of cytochalasin B (CB) and cycloheximide (CH) on the development and karyotypes of activated pig oocytes**

| Treatment | No. blastocysts with spread chromosomes | Mean number of spread chromosomes per blastocyst | N | 2N | 3N | 4N | Mixoploidy | 2N % |
|---|---|---|---|---|---|---|---|---|
| CB | 73 | 3 (1-9) | 0 | 61 | 0 | 7 | 5 | 83.6 |
| CB+ CH | 32 | 2 (1-4) | 1 | 26 | 1 | 4 | 1 | 81.3 |
| CH | 13 | 2 (1-4) | 4 | 7 | 0 | 2 | 0 | 53.8 |

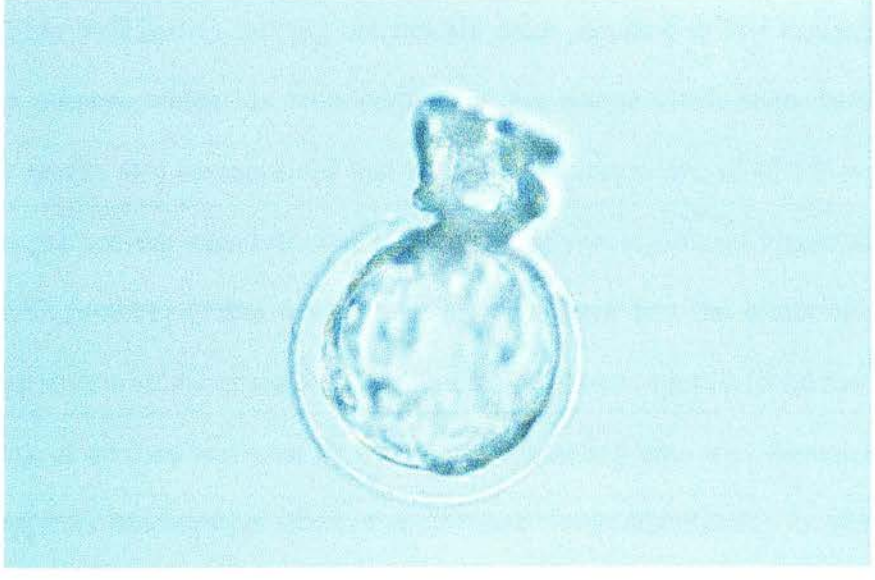
There is no significant difference in 2N% across three treatments ($p>0.05$, $\chi^2$ –Test)

**Fig 4. 3.** Parthenogenetic, hatching blastocysts produced from *in vitro* matured and cultured (**A** and **B**).

**Fig 4.3.**



**A**



**B.**

## 4.4. Discussion

### 4.4.1. Effect of activation temperature

Little information on the effect of temperature in pig oocyte activation has been reported although temperature at activation has been considered to be an important effect on developmental competence of activated oocytes. Cheng et al (1986) found that the most important factor influencing successful penetration of porcine sperm into oocytes was the temperature of incubator; less than 1% of oocytes were fertilised at 37°C but a high sperm penetration rate (89%) was achieved when sperm and oocytes were incubated together at 39°C. In addition, it is known that secondary oocytes can be very sensitive to reduction in the temperature. In sheep, oocytes cooled below 29°C for 3 h exhibited various chromosomal defects (Moor and Crosby, 1985). Moreover, in human IVF, it has been revealed that transient cooling of the secondary oocyte to room temperature can result in irreversible disruption of the meiotic spindle (Pickering *et al.*, 1990). In addition, it has been well known that pig oocytes are more sensitive to low temperature than other animals, which has been verified by pig oocyte vitrification. In this study, these results also demonstrated that the mean blastocyst rate of 43.5% was yielded when pig oocytes were activated at 37°C, which was significant higher than 32.0% at 25°C ($p<0.01$) (Table 4 1), These also indicated that the effect of activation temperature was one of major factors in pig oocyte activation. I found that when the quality of oocytes was poor or when oocyte-handling time was increased at room temperature, the negative effect of temperature would significantly increase as well. Moreover, it is known that if the intensity of the electric field is excessive, the cell

may lyse due to failure of membrane pores to reseal or osmotic swelling which excess tension in the plasma membrane (Hofmann, 1989). The temperature of the suspension medium is expected to strongly influence breakdown voltages and recovery time (Farkas, 1989). It has been verified that at a temperature closed to the growing temperature of cells, pores induced by electrical field strength tend to shrink or to reseal much faster than they would at a low temperature (Kinosita and Tsong, 1978, 1979), therefore the high temperature could promote the resealing of pores after electrical activation, which would avoid excessive $Ca^{2+}$ flowing into the oocytes as excessive $Ca^{2+}$ results in damaging oocytes. These results suggest that temperature control in oocyte activation is very important for the subsequently development of activated pig oocytes. However, increasing temperature at activation would result in enhancing activation medium vapour. Therefore, I changed activation medium frequently and handled oocytes as rapidly as I could in order to minimise damage of the oocytes due to an increase of osmolarity in activation medium.

### 4.4.2. Effect of activation media

Three common activation media were used for activating IVM pig oocytes. There were no differences in either the mean blastocyst rate or the mean number of nuclei/blastocyst among the three media ($p>0.05$). However, handling oocytes in Zimmermann's medium seemed to be more difficult than in the other two media since IVM pig oocytes became sticky and floating in the medium. In addition, it should be noted that conductivity of Zimmermann' s medium is slightly higher than those of the other two (0.5mV vs.0.25mV Sorbitol's and 0.15 mV Mannitol's),

which suggests that pig oocytes in Zimmermann's medium may not effectively be activated by higher electrical field strengths compared to in other two media. However, these data suggest that these three activation media can be used equally effectively to activate IVM pig oocytes under the conditions, but due to the problem mentioned above Zimmermann's medium seems not to be as good as the other two media.

### 4.4.3. Effect of $Ca^{2+}$ concentration in activation medium

It has been well known that $Ca^{2+}$ plays an important role in oocyte activation. The electrical pulse itself is not responsible for efficiently activating oocytes (Collas *et al*, 1993). There has been evidence showing that the presence of $Ca^{2+}$ in activation medium is essential for electrically mediated oocyte activation (Ozil, 1990; Rickords, and White, 1992; Collas and Robl, 1991). Furthermore, Liu and Moor (1997) found that the presence of $Ca^{2+}$ and $Mg^{2+}$ ions in the activation medium was necessary for the activation of *in vitro* matured pig oocytes. They also observed in the absence of both ions, no more than 15% of oocytes with pronuclear formation that was similar to the rate of spontaneous activation in pig oocytes. As a result of stimulating oocytes in the presence of $Ca^{2+}$, a flux of $Ca^{2+}$ occurs into the oocyte, resulting in a transient elevation of intracellular $Ca^{2+}$ concentration. Intracellular $Ca^{2+}$ elevations upon fertilisation are believed to be critical for resumption of meiosis and entry into the first cell cycle (Collas, *et al* 1993), whereas no spike of calcium in pig oocytes in activation medium containing no calcium was observed after electrical stimulation (Sun *et al.*, 1992). However, the calcium peak is not related to activation, the concentration of calcium in activation medium may not be

critical for activation (Robl *et al,* 1993), since in mouse and pig oocytes, a marked increase of $Ca^{2+}$ occurs even in the absence of extracellular $Ca^{2+}$, indicating that part of the $Ca^{2+}$ originates from intracellular stores (Robl *et al* 1993; Wang *et al* 1999). Interestingly, from these data above, at least two key points need to be verified: (1) whether $Ca^{2+}$ in activation medium for electrical activation is essential or not? (2) how does $Ca^{2+}$ in activation medium influence oocyte activation? In these experiments, the results clearly indicate that pig oocyte in the absence of $Ca^{2+}$ in either M or MM medium can successfully be activated and develop to the blastocyst stage, which demonstrates that $Ca^{2+}$ in the activation medium is not essential for activating IVM pig oocytes. However, MMCL yielded a highest mean blastocyst rate of 44%, which was significantly higher that those of the other three media ($p<0.05$). This result showed that $Ca^{2+}$ at a concentration of 0.05mM in the activation medium could enhance the development of activated oocytes, whereas when the concentration of $Ca^{2+}$ in the activation medium was doubled the mean blastocyst rate significantly reduced from 44 to 26% ($p=0.035$). This suggests that adequate $Ca^{2+}$ in activation medium can enhance the development of activated pig oocytes, whereas a high concentration of $Ca^{2+}$ in activation medium would be toxic to the oocytes. Therefore, IVM pig oocytes seem to be sensitive to the change of $Ca^{2+}$ concentration in the activation medium. In other words, $Ca^{2+}$ concentration in activation medium is very important for developmental potential of IVM pig oocytes activated by electrical pulses. By contrast, $Mg^{2+}$ in activation medium seems not to be important and essential for activating IVM pig oocytes as MM medium yield the lowest mean blastocyst rate although this did not significantly differ from M and MMCH media ($p>0.05$).

### 4.4.4. Effect of $Mg^{2+}$ concentration in activation medium

All current electrical activation media contain $Mg^{2+}$. Surprisingly, the results showed that MC yielded the highest mean blastocyst rate of 47.6%, which was not significantly different from those 37.9% and 42.5% of MCML and MCMH ($p>0.05$), but significantly different from 34.4% of M ($p<0.01$). These results clearly suggest that IVM pig oocytes can be activated in activation medium containing no $Mg^{2+}$ and that the activated oocytes are able to develop to the blastocyst stage. Moreover, based on the data in the last experiment, MC medium gave the highest mean blastocyst rate, indicating that $Mg^{2+}$ in activation medium is absolutely not necessary for IVM pig oocyte electrical activation. In the presence of $Ca^{2+}$, addition of $Mg^{2+}$ in the activation medium at the concentration of either 0.05 or 0.1 mM seemed not to significantly affect the mean blastocyst rate of activated oocytes. Therefore, $Mg^{2+}$ has no positive effect on the blastocyst rate of electrically activated IVM pig oocytes compared to $Ca^{2+}$.

In summary, it should be noted here that in the last two experiments, although pig oocytes were activated in the activation medium M (which had no $Ca^{2+}$ and $Mg^{2+}$), this does not prove that activation can occur without $Ca^{2+}$. The source of $Ca^{2+}$ could originate from two possible ways: (1) a release of $Ca^{2+}$ intracellular stores in the oocytes might be triggered by the electric pulses; (2) but it is more likely that a $Ca^{2+}$ increase in the oocytes could occur due to a flux of extracellular $Ca^{2+}$ (from the culture medium containing a high concentration of $Ca^{2+}$) into the oocytes through electrical field strength - induced pores after electrical pulses when the oocytes were placed into the culture medium since the resealing of the pores

would last tens of minutes after electrical pulses (Collas *et al.,* 1993). This phenomenon has been mentioned in Chapter 1. Thus, the question of whether or not extracellular $Ca^{2+}$ is essential for oocyte activation remains unanswered in these experiments. Further experiments apparently need to be carried out in order to verify them.

### 4.4.5. Effect of cytochalasin B treatment

It is confirmed that cytochalasin B, microfilament inhibitor can suppress the extrusion of the second polar body. Thus, cytochalasin B is commonly used to treat activated oocytes or cloned embryos in order to protect normal diploid karyotype, which is thought to be more important for the developmental competence of these cells. Kure-bayashi, *et al* (1996) reported that 91% of activated oocytes that had not been treated with cytochalasin B were haploid after activation by a single pulse and that 92% the oocytes treated with 5.0 μg/ml cytochalasin B for 4 h were diploid. Also, their results showed that the blastocyst rate in diploid oocytes was significantly higher than that in haploid ones ($p<0.01$). Likewise, other results (Cha *et al*, 1997; Kaufmann and Sachs, 1976; Kim *et al*, 1997) also confirmed that diploid parthenogenetic oocytes had a great developmental potential compared with haploid parthenotes. In this experiment, the data supported this conclusion and showed that with the time of CB treatment, the proportion of diploid oocytes, the mean blastocyst rate on day 7 and the percentage of diploid blastocysts increased (Table 4.3 and Table 4.4.). Interestingly, the mean blastocyst rate significantly increased from 21.1 to 39.8% during the first 3 h of CB treatment ($P<0.01$), whereas the rate increased only from 41.9 to 42.5% during the period between 4 and 6 h ($P>0.8$)

meanwhile the proportion of the diploid blastocysts increased from 39.8 to 73.3% during the first 3 h, but only from 76.2 to 84.2% during the period of the last 3 h. Therefore, these indicate that 3 h seem to be a minimum duration of CB treatment for the reasonable development and diploid blastocyst rates of activated IVM pig oocytes. However, the percentage of diploid oocytes seems to increase with the time of CB treatment. The percentage reached 97.2% at 6 h of CB treatment. The correlation coefficient ($R_2$) between the mean proportions of diploid oocytes and the mean proportions of diploid blastocysts was 0.902 ($p<0.05$) and the correlation coefficient ($R_1$) between the proportions of diploid blastocysts and the mean blastocyst rates was 0.959 ($p<0.01$), suggesting that CB treatment did highly influence the karyotype and developmental potential of the activated oocytes. Surprisingly, no differences in the mean number of nuclei/blastocyst were observed among the treatments ($p>0.05$), which differed from other observation (Kure-bayashi *et al*, 1996) that the mean number of nuclei/blastocyst in diploid blastocysts were significantly higher than those in haploid blastocysts ($p<0.01$). In addition, the proportion of diploid oocytes (26.5%) at 0 h of CB treatment was higher than 9% in the previous report in which a single electrical pulse was used (Kure-bayashi *et al*, 1996). This difference may be due to the use of different pulse numbers since using multiple pulses could result in an increased percentage of diploid parthenotes (Grupen *et al*, 1999) compared to a single pulse or due to the different culture medium used. Clearly, the further research needs to be done.

### 4.4.6. Effect of CB and cycloheximide

Treatment with cycloheximide, a protein–synthesis inhibitor after parthenogenetic stimulation has been shown to increase activation rate in mice (Clark and Masui 1983), in cattle (Yang *et al*, 1994; Presicce and Yang 1994) and pig oocytes (Nussbaum and Prather 1995). It has been suggested that the inhibitor of protein synthesis prevent synthesis of cytostatic factor, which seems to be linked with maturation promoting factor (MPF) (Yang *et al*, 1994), since high levels of MPF are known to be essential for meiotic arrest of mammalian oocytes, its breakdown would increase the incidence of activation after parthenogenetic stimulation (Cha *et al*, 1997). The results from the present experiments showed that treatments with CB alone for 6 h and CB + CH for 4 h after electrical stimulation resulted in the similar mean blastocyst rates (45.7 vs. 42.9%), percentages of diploid blastocysts (83.6% vs. 81.3%) and the mean number of nuclei/blastocyst (41.9±15.8 vs. 38.1±12.9) ($p>0.05$). Therefore, both these treatments are suitable for activated IVM pig oocytes. By contrast, cycloheximide alone seemed not to enhance the mean blastocyst rate of activated oocytes (17.3%) or to significantly improve the percentage of diploid blastocysts (53.8%) compared to CB or CB + CH treatments. However, the mean number of nuclei/blastocyst in CH treatment (41.8 ± 20.5) was comparable to those in the other two treatments. Thus, the treatment with CH alone is not beneficial for improving pig oocyte activation after electrical activation. These results are similar to the previous observation (Cha *et al*, 1997).

In summary, these data from this chapter show that temperature at activation is critical for successful pig oocyte activation. Three activation media tested are suitable for pig oocyte activation, but Zimmermann's medium causes a difficulty to handle oocytes. Pig oocytes can electrically be activated in activation medium

containing no $Ca^{2+}$ and $Mg^{2+}$, and develop to the blastocyst stage. An adequate concentration of $Ca^{2+}$ in activation medium enhances the development of activated IVM pig oocytes, whereas a high concentration of $Ca^{2+}$ in activation medium could be toxic to the oocytes. $Mg^{2+}$ in activation medium is not essential and beneficial for electrically activating IVM pig oocytes. CB treatment of 3 h after electrical activation would significantly increase the mean blastocyst rate of activated oocytes with over 70% of the blastocysts being diploid. Activated IVM pig oocytes are treated with CB for 6 h, which results in diploid oocytes of 97.2%. In addition, CB + CH treatment of 4 h following electrical activation can achieve the same mean blastocyst rate, percentage of diploid blastocysts and mean number of nuclei/blastocyst as CB alone treatment for 6 h. However, CH alone is not beneficial for pig oocyte electrical activation.

# Chapter 5

# *In Vivo* Developmental Competence of Electrically Activated Porcine Oocytes

## 5.1. Introduction

In the last chapter, the parthenote blastocyst rate and quality of the parthenote blastocysts have been improved. However all the work was carried out *in vitro*. Developmental competence can only be assessed by monitoring development *in vivo*. Mature oocytes can be artificially activated by a variety of chemical and physical stimuli in the pig (Sun *et al*, 1992; Cha, *et al,* 1997;Wang *et al*, 1998; Leal and Liu, 1998; Machaty *et al* 1995; Hagen *et al*, 1991), and can develop *in vitro* to the blastocyst stage (Grupen *et al*, 1999; Koo *et al*, 2000; Cha *et al*, 1997). Oocytes activated by different protocols may not have equally developmental competencies (Wang *et al*, 1998; Loi *et al*, 1998). Oocyte activation is a vital step of the nuclear transfer procedures. Therefore, to assess *in vitro* or *in vivo* developmental competence of activated oocytes is essential for evaluating activation protocols for nuclear transfer. In addition to oocyte activation for nuclear transfer, parthenogenetic development can also be used to study imprinted genes of either maternal or paternal loci. Parthenogenetic development beyond the blastocyst stage in mouse (Kono *et al*, 1996), rabbit (Ozil, 1990), sheep (Loi *et al*, 1998), cattle (Fukui *et al*, 1992) and pig (Kure-bayashi *et al*, 2000; Zhu *et al,* 2000) have been reported. From these studies, it has been confirmed that parthenogenetic fetuses can

survive *in vivo* for 9 days in mouse, 12 days in rabbit, 21 days in sheep, 30 days in cattle and 29 days in pigs.

A blastocyst rate of over 40% could be achieved in porcine oocytes activated by the improved activation protocol and that over 80% of those would be diploid (See Chapter 3). The objectives of these experiments were to (1) evaluate this current activation protocol for pig nuclear transfer; (2) to determine *in vivo* developmental competence of parthenogenetic embryos in pigs. Here, I report that using this activation protocol pig parthenogenetic embryos can develop *in vivo* for at least 30 days and that they stop development around day 31 of gestation.

## 5.2. Material and Methods

### 5.2.1. Oocyte collection and maturation

Pig ovaries were collected from slaughtered gilts at a local slaughterhouse and transported to the laboratory in Dulbecco's phosphate-buffered saline (PBS) at 25–30°C. Ovaries were rinsed three times in PBS. They were then stored in a water bath at 25–30°C. Cumulus oocyte complexes (COCs) were aspired from ovarian follicles 3-8mm in diameter using an 18-gauge needle attached to 10 ml syringe. Subsequently, COCs were rinsed three times in Hepes-buffered–TL containing 0.1% (w/v) polyvinyl alcohol (Hepes-TL-PVA). Oocytes with more than three layers of compact cumulus cells were selected for maturation. The selected COCs were washed three times in maturation medium containing NSCU23 medium, 10% pig follicular fluid (pFF), 0.6 mM cysteine, 1% (v/v) essential amino acids (sigma) and 0.5% (v/v) non-essential amino acids (Sigma). After three washes, 50-60 COCs

were transferred into 500 µl drops of maturation medium containing 10 IU/ml eCG and 10 IU/ml hCG (both from Intervet, Cambridge, UK) in 4 well dishes and cultured for 22 h at 39°C in 5% $CO_2$ in air. Afterwards, COCs were washed three times in the maturation medium without hormonal supplements and transferred into 500 µl drops of the same medium and cultured for an additional 22-h.

Ovulated oocytes were produced from large-white gilts that were approximately 9 months of age or older and weighted at least 120 kg at time of use. Superovulation was carried out by a protocol described by Dobrinsky *et al* (2000), after 41-48 h following hCG injection, oocytes were surgically recovered following a general anaesthetic and mid-line laparotomy by flushing the oviducts with prewarmed (38°C) sterile, Hepes - buffered NCSU23 medium containing 0.4% BSA-V.

### 5.2.2. Electrical activation of pig oocytes

After 43-44 h of maturation, cumulus cells of IVM pig oocytes were removed by repeated pipetting in the 4-well dishes, only denuded oocytes were selected for activation. Ovulated or IVM oocytes were rinsed twice in $Ca^{2+}$ free-NCSU23 medium containing 0.4% BSA and twice in activation medium containing 0.3 M Mannitol, 0.1 mM $Mg^{2+}$ and 0.05 mM $Ca^{2+}$. Oocytes were then activated by 3 x 80 µsec. pulses of 1.0 kV/cm DC following 0.25 kV/cm AC for 5 sec using a fusion machine (CF-150/B series, BLS-Ltd, Hungary). All manipulation of the oocytes was carried out on a warm stage at 37°C. After activation, the oocytes were directly transferred into 7.5 µg/ml cytochalasin B in NCSU 23 medium supplemented with 0.4% BSA and cultured for 6 h at 39°C in 5% $CO_2$ in air. Finally,

30-40 activated oocytes were cultured in 500 µl drops of NCSU 23 medium containing 0.4% BSA in 4-well dishes covered with mineral oil (Sigma) for 1 day, 2 days or 6-7 days, which depended on the requirement of the experiment, at 39°C in 5% $CO_2$ in air.

### 5.2.3. Collection of fertilised embryos and embryo transfer

Fertilised oocytes were used as a control in this study. Mature gilts as embryo donors were mated twice 6 h apart with a mature large-white boar following natural heat (oestrus day = day 0). In experiments 1 and 2, fertilised embryos were collected on day 2 following mating, whereas in experiment 4, fetuses were recovered on day 21 and 30.

Fertilised embryos and/ parthenotes were transferred following a mid-line laparotomy under general anaesthesia. During surgery, the reproductive tract was exposed and embryos and/or parthenotes were transferred into the oviduct using a 3.5 cm French gauge tomcat catheter on day 1, day 2 or day 3 gilts that exhibited a natural synchronous heat. The type of the recipients used depended on the experiment.

### 5.2.4. Pregnancy monitoring

All recipient animals underwent a trans-abdominal ultrasound examination using an Aloka 500SD ultrasound machine (Made in Japan) with a 5 MHz convex liner probe between day 21 and 23 post-oestrus.

A result was classed as positive if a fluid filled area containing a fetal mass could be detected. A negative result was confirmed when no fluid filled areas in the

uterus could be detected. Ultrasound examination was repeated at regular intervals until two consecutive negative results were obtained at which point the gilt was classed as non-pregnant. All recipients were examined using ultrasonography, twice weekly from day 21 (Oestrus = day 0).

### 5.2.5. Blastocyst staining with Hoechst 33342

Day 6 or day 7 blastocysts were washed twice in Hepes-buffered NCSU 23 with 0.4% BSA, they were then transferred into 50 μl droplets of the same medium containing 5 μg/ml Hoechst 33342 (Sigma) and cultured in the incubator for 10 min. After incubation, blastocysts were gently mounted on a cleaned glass slide and covered with a cleaned glass coverslip. These slides were immediately observed under UV light or stored in a fridge at 4°C until observation.

### 5.2.6. Collection of fertilised and parthenogenetic fetuses

Fertilised or parthenogenetic fetuses were recovered following euthanasia of pregnant recipients. Fetuses surrounded by placentas were gently separated from the uterus using two pairs of forceps. Subsequently, fetuses were removed from their placentas and placed on a piece of clean, dry tissue using a pair of forceps in order to dry liquid on the surface of the fetuses, its crown rump length and body weight were recorded. In addition, the morphology of parthenogenetic fetuses was compared with that of fertilised fetuses collected at the same stage.

## 5.3. Statistical Analysis

Data collected from the experiments were analysed by a computerized programme Excel, Student Test or $\chi^2$–Test. If p value is less than 0.05, it is considered to be a significant difference.

## 5.4. Experimental Design:

### 5.4.1. Experiment. 1: A comparison of *in vitro* development of activated IVM and ovulated pig oocytes

The aim of this experiment was to determine whether the current electrical activation protocol would be suitable for activating both ovulated and IVM pig oocytes. 44 h post matured and ovulated pig oocytes were electrically activated by the standard method, and cultured *in vitro* for 7 days. Fertilised embryos collected on day 2 following service were used as a control and cultured *in vitro* for 5 days. Blastocysts in the three groups were stained by Hoechst 33342 and nuclei were counted in the blastocysts under UV lights.

### 5.4.2. Experiment 2. *In vivo* development of IVM parthenotes on day 21

This experiment was designed to assess the potential of *in vivo* development of IVM parthenotes. IVM pig oocytes were electrically activated and cultured overnight, 58-60 activated oocytes were surgically transferred into the oviducts of synchronous gilts. The parthenotes were collected on day 21. Fertilised embryos surgically recovered from naturally mated gilts on day 2 (Oestrus = day 0) were used as a control, and 20 collected embryos were transferred to a synchronous recipient

within 3 h. Fertilised fetuses were also collected on day 21. Four recipients were used for this experiment.

### 5.4.3. Experiment 3. *In vivo* developmental loss of IVM parthenogenetic embryos

55-60 activated oocytes, which were cultured overnight post activation, were transferred into 13 recipient gilts that exhibited nature heat 2 days before transfer. Pregnancy was determined by an ultrasound examination at day 21–23 of gestation; afterwards the pregnant animals were scanned by an ultrasound machine twice a week until the fetuses could not be detected.

### 5.4.4. Experiment 4. *In vivo* development of ovulated parthenotes

The experiment was carried out to assess *in vivo* development of ovulated parthenotes. Ovulated oocytes were surgically collected from superovulated gilts 41-48 h after the hCG injection. Oocytes were electrically activated by the standard protocol and cultured for 48 h. Only cleaved 2-4 cell embryos were transferred to synchronous gilts. Pregnancy was determined by an ultrasound examination on day 21 and before surgery. Fetuses were surgically collected on day 30 (Oestrus = day 0). A total of four recipients were used for this experiment.

### 5.4.5. Experiment 5. *In vivo* developmental competence of IVM parthenotes.

The objective of this experiment was to improve the fetal developmental rate of IVM parthenotes and to determine when parthenotes would stop development. IVM oocytes were electrically activated and cultured *in vitro* for 2 days. Only

cleaved oocytes were randomly taken and surgically transferred into recipient gilts that had exhibited heat 2 or 3 days before. Each animal received 35–48 cleaved parthenotes. Fetuses were surgically collected on day 21, 30 and 35, respectively. Fertilised fetuses collected from naturally mated gilts on day 21 and day 30 were used as controls, respectively. A total of 17 animals including four day 3 and thirteen day 2 synchronous recipients were used for IVM parthenote transfer in this experiment.

## 5.5. Results

### 5.5.1. A comparison of *in vitro* development of activated IVM and ovulated pig oocytes

In experiment 1, there was a tendency for more blastocysts to be formed from fertilised embryos (84%) than from *in vivo* derived parthenotes (61%) which in turn formed more blastocysts than *in vitro* produced parthenotes (46%) (Table 5.1). However, the only significant difference was between fertilised embryos and IVM activated oocytes that were cultured *in vitro* for 7 days ($p<0.01$; Table 5.1) There was no significant difference in the mean blastocyst rate either between fertilised embryos and ovulated parthenotes (84 vs. 61%; 50/82) or between fertilised embryos and IVM parthenotes (61 vs. 46%; $p>0.05$). Likewise, there were no differences in the mean number of nuclei/blastocyst across three treatment ($p>0.05$).

### 5.5.2. *In vivo* development of IVM parthenotes on day 21

In experiment 2, two from 3 recipients that had received fertilised embryos became pregnant, as determined by ultrasound examination on day 21, and 3 from 4 that had received IVM parthenotes were pregnant. The mean crown-rump length in naturally mated fetuses (11.26 ± 1.33 mm, n=25; Table 5.2.) was significantly longer than that in the mated, transferred ones (10.19 ± 2.15 mm, n=35) ($p<0.05$) although there was no difference in the mean wet body weight between these two groups (151.53 ± 34.77 mg vs. 132.63 ± 71.49 mg) ($p>0.05$). In addition, parthenogenetic fetuses were significantly smaller (7.75 ± 1.06 mm, n=16) and lighter (57.36 ± 16.92 mg) than both naturally mated or the mated, transferred fetuses ($p<0.01$). However, 14 parthenogenetic fetuses had the same morphology as the fertilised controls (Fig 5.1.)

### 5.5.3. *In vivo* developmental loss of IVM parthenogenetic embryos

The result was shown in Table 5.3. Eight of 13 transferred animals were pregnant at day 21–23. Of which, 7 pregnancies were lost (could not be detected on scans) between 41 and 54 days of gestation, one pregnancy was lost on day 28 due to cystic follicles seen on a later scan. The pregnancy rate of IVM pig parthenotes at day 21 was 63%.

### 5.5.4. *In vivo* development of ovulated parthenotes

One from 4 transferred recipient animals was pregnant (Table 5.4), 16 fetuses were surgically collected on day 21. The mean crown-rump length was 14.53 ± 6.34 mm. 7 of 16 fetuses were larger than 20 mm (20.8 to 22.6 mm), 4 (10.1-12.6

mm) and 5 smaller than 10 mm (6.4-9.5 mm). Normal morphology in the large fetuses was observed.

### 5.5.5. *In vivo* developmental competence of IVM parthenotes

In experiment 5, parthenotes were transferred to the first 4 recipients on day 3 (Oestrus=day 0) but none of them became pregnant. Subsequently, recipients were used on day 2 when 11 of 13 recipients became pregnant (85%) (Table 5.5). Fetuses were surgically collected from 4 pregnant recipients (day 21), 5 (day 30) and 2 (day 35). The fetal implantation rates (fetuses/transferred parthenotes) were 20% in recipients killed on day 21 (32/160), 16.4% on day 30 (37/226) and 28.6% on day 35 (20/70). (Table 5.5.). Beating hearts were still observed in some fetuses during ultrasound examinations on day 31 of gestation. However, there was no heartbeat detected in any of the 20 fetuses collected on day 35. Additionally, there was a significant difference in the fetal implantation rate between day 30 and day 35 parthenote fetuses ($p<0.05$, $\chi^2$-Test), but no difference either between day 21 and day 30 or between day 21 and day 35 ($p>0.05$) was observed (Table 5.5). Moreover, A wide range of both the crown-rump length and the wet body weight was observed in all the parthenote groups compared to those in the mated controls (Table 5.6). Likewise, there was a large variation of the crown-rump length and the wet body weight across individual animals, even in the same treatment (data not shown). The majority of day 21 and day 30 fetuses had the same or similar morphology compared with the controls at the same developmental stage such as pigment forelimb bud hind limb bud, IVth ventricle, fore brain vesicle, facial sinuses and liver, heart, head and eyes. In addition, one largest day 35 foetus had slightly advanced morphology

than those of both day 30 parthenogenetic and mated control fetuses (Figs 5.2, 5.3 and 5.4) although its crown-rump length and wet body weight were similar to those of the large day 30 pig parthenotes. However, abnormalities in some tissues, organs such as limb bud, otic, optic lens, brain differentiation, nose, eyelids, liver and heart as well as defect development of head, eyes and mouth regions were externally visible in some parthenotes, especially on day 30 and 35.

**Table 5.1. *In vitro* development of activated oocytes from either ovulation or *in vitro* maturation**

| Cell type | No of used oocytes or embryos | No. of blastocysts on day 6 | Blastocyst rate (%) Mean ± SD | Mean no. of nuclei/ blast. Mean ± SD |
|---|---|---|---|---|
| *Fertilised embryos | 85 | 71 | 82.4±11.3[a] | 32.8±11.8 |
| **In vivo* matured oocytes | 82 | 50 | 61±10.2 | 27.1±7.7 |
| *In vitro* matured oocytes | 275 | 126 | 46.2±12.1 [b] | 25.1±10.9 |

*The data collected from four replicates.

There is a significant difference between *a* and *b* ($p < 0.01$; T- Test).

**Table 5.2. Comparisons of the mean crown-rump length and the mean wet body weight among mated, mated-transferred and parthenogenetic fetuses**

| Type of fetus | No. of transferred embryos | No of recipients | No. of fetuses on day 21 | Crown-rump length (mm) Mean ±SD | **Wet. body weight Mean ±SD (mg) |
|---|---|---|---|---|---|
| Naturally mated | - | 3 | 25 | $11.26\pm1.33^{ad}$ | $151.53\pm34.77^{d}$ |
| Naturally mated and transferred | ***61 | 3 | 35 | $10.19\pm2.15^{bd}$ | $132.63\pm71.49^{d}$ |
| IVM parthenotes | ***239 | 4 | 16 | $7.75\pm1.06^{c}$ | $57.36\pm16.92^{c}$ |

*: The mean length of crown-rump (mean ± SD).
**: The mean wet. body weight (mean ± SD).
*** The number of transferred embryos represents a total number of transferred, activated oocytes in all recipients including non-pregnant or pregnant.
Note: there is a significant difference between either *a* and *b* ($p < 0.05$; Student's T-Test); or *c* and *d* ($p < 0.01$; Student's T-Test).

**Table 5.3. Pig parthenotes developing *in vivo***

| Pig ID | Surgery date | No of transferred oocytes | 1st. Scanning results | pregnancy loss date | No. of days till pregnancy loss | Comments |
|---|---|---|---|---|---|---|
| 6350 | 17/3/00 | 60 | positive | 2/May | 49 days | |
| 6219 | 17/3/00 | 58 | negative | 5/April | <21 days | |
| 6122 | 24/3/00 | 60 | negative | 14/April | <21 days | |
| 6336 | 24/3/00 | 55 | negative | 14/April | <21 days | |
| 6671 | 31/3/00 | 60 | positive | 17/May | 50 days | natural heats |
| 6289 | 31/3/00 | 60 | negative | 19/April | <21 days | natural heats |
| 6831 | 7/4/00 | 60 | positive | 28/May | 54 days | natural heats |
| 6139 | 7/4/00 | 60 | positive | 17/ May | 42 days | natural heats |
| 7100 | 7/4/00 | 60 | positive | 3/May | 28 days* | natural heats |
| 7116 | 13/4/00 | 59 | negative | 21/May | <21 days | natural heats |
| 6911 | 13/4/00 | 60 | positive | 21/May | 41 days | natural heats |
| 6615 | 13/4/00 | 59 | positive | 28/May | 48 days | natural heats |
| 6434 | 20/4/00 | 60 | positive | 2/June | 52 days | natural heats |

Note: Natural heats also cystic follicles on a later scan. The first four recipients were treated by artificial synchronisation with hormonal treatments. The first scanning was carried out on day 21 or day 23. Subsequently, examinations were carried out twice weekly

**Table 5. 4. *In vivo* developmental of ovulated, activated pig oocytes.**

| Replicates | No oocytes | No. of Activated oocytes | 48h after activation | | | | No. of transferred parthenotes On day 2 | No. of fetuses on day 30 |
|---|---|---|---|---|---|---|---|---|
| | | | 1-cell | 2-cell | 4-cell | fragmented | | |
| 1 | 245 | 237 | 87 | 18 | 82 | 9 | 60 | 0 |
| 2 | 130 | 102 | 47 | 5 | 48 | 2 | 48 | 0 |
| 3 | 132 | 129 | 53 | 11 | 56 | 2 | 54 | 16* |
| 4 | 94 | 81 | 43 | 16 | 24 | 1 | 24 | 0 |

The mean crown-rump length in 16 fetuses is 14.53±6.34 mm, including 7 fetuses > 20 mm (20.78-22.55mm), 4 fetuses between 20 and 10 mm (10.08-12.62 mm) and 5 fetuses < 10mm (6.40-9.47mm).

**Table 5.5. *In vivo* developmental competence of IVM parthenote fetuses**

| Item | Day 21 | Day 30 | Day 35 |
|---|---|---|---|
| Pregnant animals (N) | 4 | 5 | 2 |
| A total of transferred cleaved oocytes (N) | 160 | 226 | 70 |
| No. of collected fetuses | 32 | 37 | 20 |
| Fetal implantation rate (%) | 20 | $16.4^{a}$ | $28.6^{b}$ |

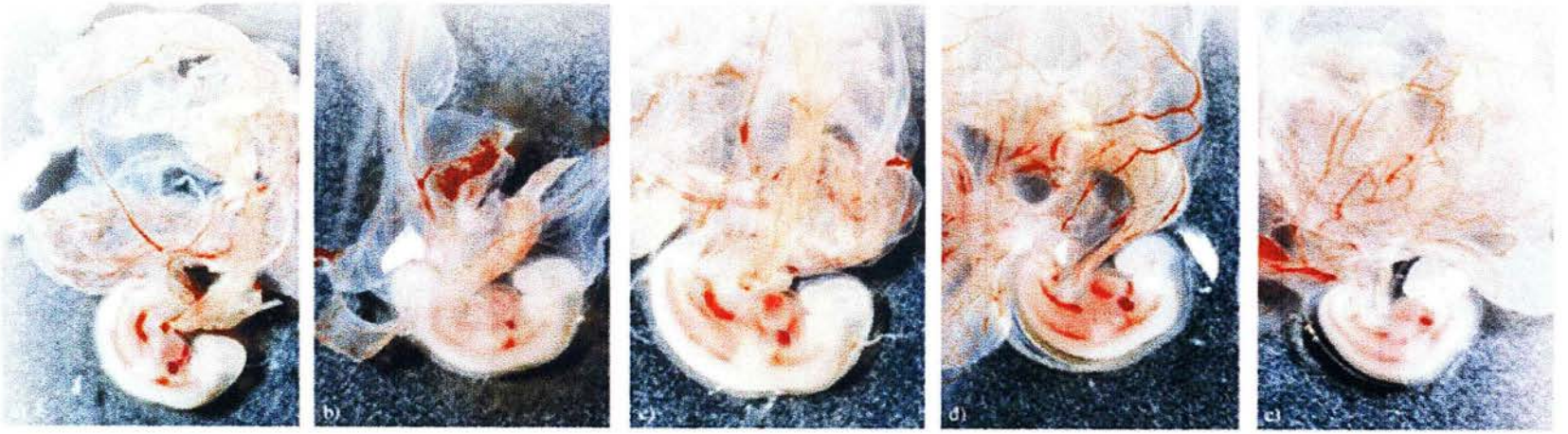
There is a significant difference between *a* and *b* ($p < 0.05$; $\chi^2$- Test).

**Table 5.6. Comparisons of fertilised fetuses and IVM parthenogenetic fetuses**

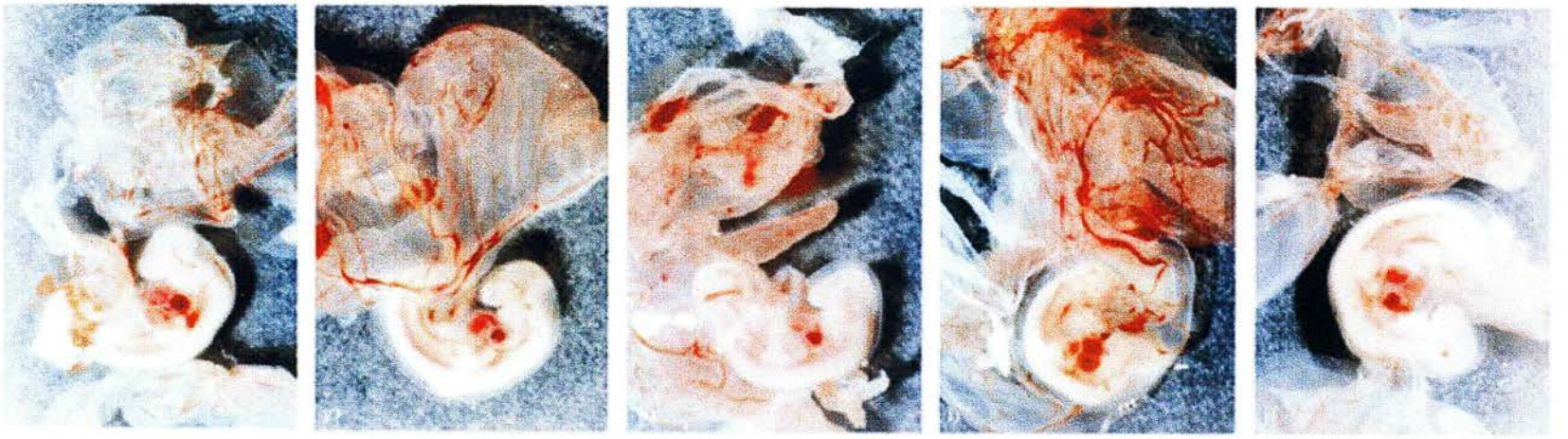
| Item | No. of foetuses | Crown- rump length (mm) | | | Wet. body weight (mg) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | >20 | 20-10 | <10 | >1000 | 1000-500 | 500-100 | 100-50 | <50 |
| Day 21 control | 13 | | 7(10.5-10) | 6 (9.5-8.5) | | | 8 (161-104) | 5 (99-69) | |
| Day 21 parthenotes | 32 | | 1 (11) | 31(8-4) | | | | 10 (96-55) | 22 (4.8-8.3) |
| Day 30 control | 7 | 7 (28-26) | | | 7 (1620-1892) | | | | |
| *Day 30 parthenotes | 37 | 14 (26-20) | 17 (19-11) | 6 (6.5-4) | 1 (1566) | 20 (835-512) | 10 (490-115) | 4 (84-70) | 1 (29) |
| Day 35 parthenotes | 20* | 5 (24-20) | 11 (19-15) | 4 (8-4) | 2(1350-1124) | 6 (781-515) | 8 (487-265) | 1 (87) | 3 (16.5-7) |

All fetuses died when they were collected.
* lost the data of one foetus on body weight.

**Fig 5.1.**

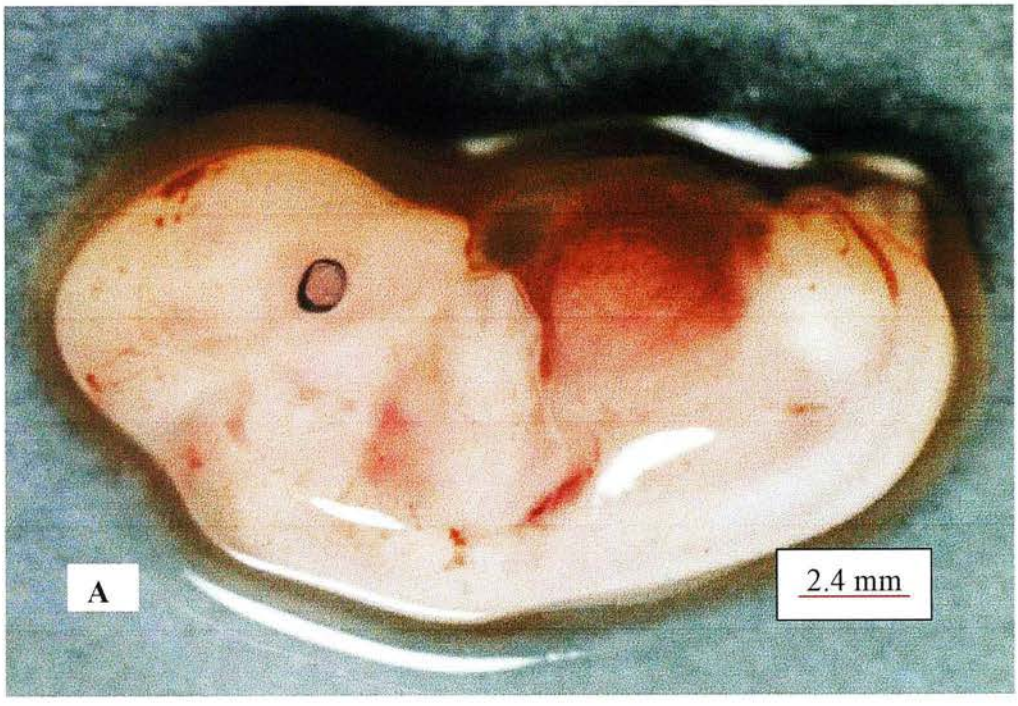


*Pictures from A to E show developmental morphology of day 21 foetuses from transferred fertilized embryos developing in vivo.*



*Pictures from F to J show developmental morphology of day 21 foetuses from parthenotes.*

**Fig 5.2. Picture A and B:** two day 30 pig parthenote fetuses from *in vitro* maturation.


A
2.4 mm

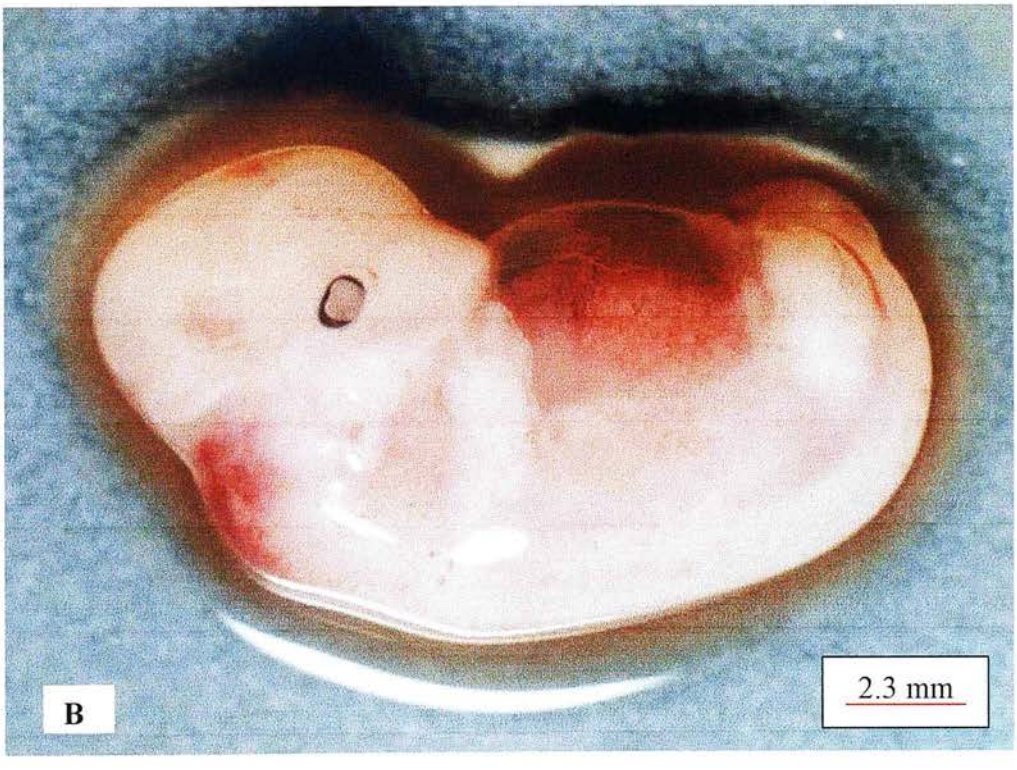
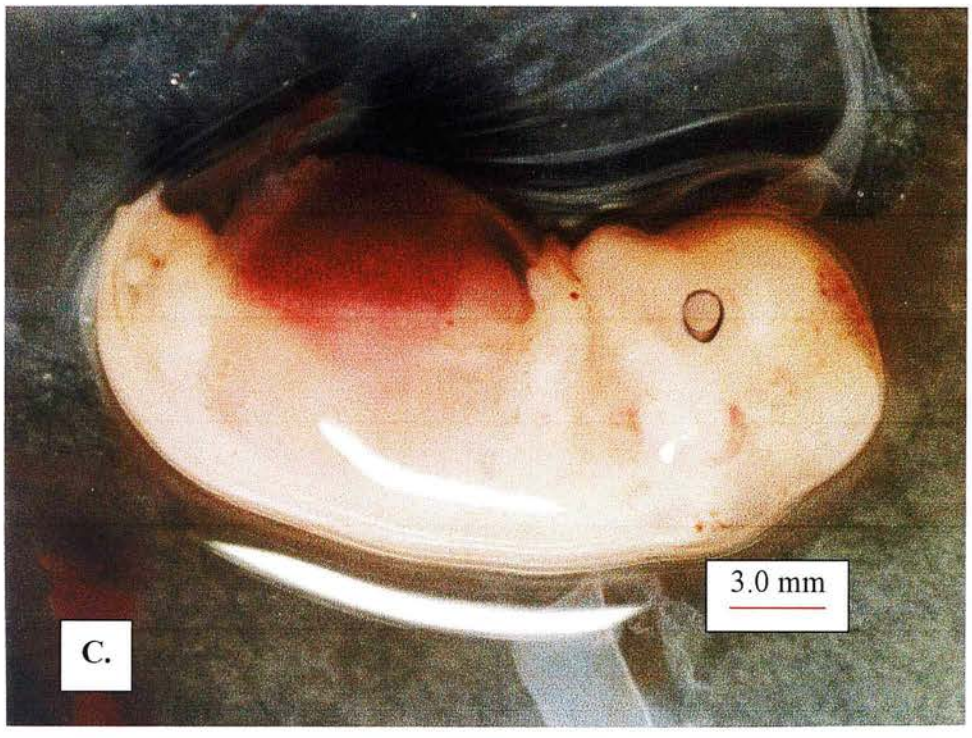

B
2.3 mm

**Fig 5.3. Picture C and D:** two-day 30 fertilised fetuses from mated gilts.


3.0 mm
C.

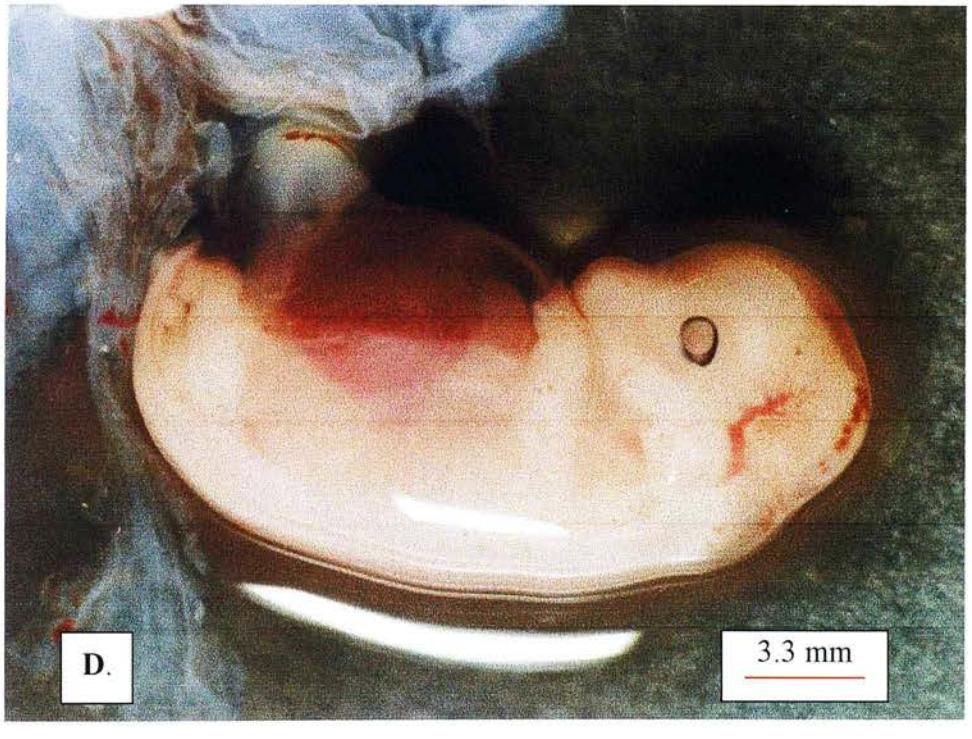

3.3 mm
D.

**Fig 5.4.** An IVM pig parthenote fetus collected on day 35 whose beat heart was seen on ultrasound scans at day 31 of gestation.


2.4 mm

## 5.6. Discussion

In this study, the data demonstrate that the electrical activation protocol is not only suitable for IVM oocytes but also effective for ovulated porcine oocytes, and that parthenogenetic porcine embryos can develop *in vivo* up to day 30, which extends the previous observations (Kure-bayashi *et al*, 2000;). Additionally, *in vivo* developmental competence of parthenogenetic porcine embryos has primarily been determined for the first time by this study. Importantly, this study also implies an alternative method to maintain a pregnancy in pigs, which may be useful for porcine nuclear transfer.

### 5. 6.1. A comparison of *in vitro* development of activated IVM and ovulated pig oocytes

Experiments described in Chapters 3 and 4 showed that the improved electrical activation protocol employed could result in a blastocyst rate of over 40% with IVM pig oocytes after activation and culture *in vitro* for 7 days. In this experiment the protocol has also been verified to be suitable for activating ovulated pig oocytes. 61% of ovulated, activated pig oocytes developed to the blastocyst stage although this did not significantly differ from that of either activated IVM (46%) or fertilised and cultured ones (83.1%) ($p>0.05$). These results indicate that the quality of the ovulated oocytes is generally better than that of *in vitro* matured ones, which has been confirmed by other observations as well (Onishi *et al*, 2000; Grupen *et al*, 1999). Therefore, although this oocyte maturation system has led to over 90% of matured oocytes at the MII stage after 44 h of maturation and over 40% of activated IVM oocytes could develop to the blastocyst stage after 7 days of

culture, this maturation system apparently still needs to be improved compared with *in vivo* matured oocytes.

### 5.6.2. *In vivo* development of IVM parthenotes on day 21

This observation on *in vivo* development of activated IVM pig oocytes shows that parthenogenetic fetuses are significantly smaller and lighter than normal and fertilised, transferred fetuses ($p<0.01$) (Table 5.2), which is similar to a previous observation (Kure-bayashi *et al*, 2000) that parthenotes on day 24 post activation were only about half the size of the control fetuses. Loi *et al* (1998) also reported that parthenogenetic sheep fetuses (N = 6) were significantly smaller than controls (N=7) fetuses (4.5 ± 0.5 mm vs. 5.2 ± 0.3 mm; $P<0.01$). In addition, in mouse parthenogenetic fetuses are also significantly smaller than normal fetuses (Surani, 1991), which is similar to the observations above. The difference in size between fertilised and parthenogenetic fetuses is due to the latter lacking insulin-like growth factor 2 (IGF2) or other paternal imprinted genes, which are only contributed from paternal side. It is well known that an extreme consequence of imprinting is that parthenogenetic embryos are small and die at early postimplantation stages because of the lack of paternally expressed genes (Surani *et al* 1984, McGrath and Solter, 1984, Hall 1990). In analogy with studies in the mouse, where genetic ablation of the Igf2 gene leads to reduced fetal growth (DeChiara *et al,* 1991), it seems likely that the absence of IGF 2 expression in parthenogenetic sheep fetuses is involved in their growth retardation (Feil *et al* 1998). In contrast, androgenetic fetuses would be bigger than normal fetuses since IGF2 is over-expression in androgenetic fetuses (Lau *et al,* 1994). In addition to IGF2, lack of expression of some other paternal

imprinting genes were also found to contribute the smaller size of parthenogenetic fetuses such as mouse and sheep PFG1/MEST (Kotzot *et al.* 1995; Feil *et al* 1998). It is worth noting that inadequate culture environment and synchronisation between transferred embryo and the recipient, even for a short period, may contribute to the smaller size of fertilised foetuses, as the culture and transfer of the fertilised embryos resulted in a significant reduction of their mean crown-rump length ($p<0.05$) but not body weight compared with the mated controls at day 21. However, this difference may decline and disappear with time of gestation. Further investigation on the detailed mechanism(s) certainly needs to be done, especially at the level of molecular biology.

### 5.6.3. *In vivo* developmental loss of IVM parthenogenetic embryos

This experiment demonstrates for the first time *in vivo* developmental loss of IVM parthenogenetic porcine embryos. Although the exact date of parthenogenetic fetal death was not identified in this experiment, the data show that most fetuses would be non-detectable by ultrasound scanning between 40–50 days of gestation (Table 5.3), which directly supports the hypothesis that parthenogenetic embryos as potential helpers could enhance a pregnancy in pigs since the parthenogenetic fetuses could appear in the uterus of recipient animals beyond day 30 that is regarded to be a critical stage for a pig pregnancy, since porcine embryos signal the mother around 9-11 days and their implantations start to take place around 12–15 days of gestation. This result implies that parthenogenetic IVM pig fetuses may die between 30 and 40 days of gestation. The designs of experiments 4 and 5 were based on this observation. The pregnancy rate of IVM pig parthenotes in this experiment

reached 63%, which was comparable to the normal pregnancy rate of 60–75% reported after surgical transfers (Cameron et al., 1989). It should be noted here that the variation among recipient animals could be a main effect on the pregnancy rate in this experiment since the same numbers of activated oocytes that were believed to be same quality were transferred into different recipients at the same days, some became pregnant and some not. Furthermore, the first four animals used were treated by hormones to synchronise activated oocytes and recipients, only one from four animals became pregnant, which was lower than that of the recipients that were synchronised by natural heats. Afterwards, all recipient animals used were synchronised by natural heat.

### 5.6.4. *In vivo* development of ovulated parthenotes

In experiment 4, parthenotes were cultured *in vitro* for 2 days, only 2–4 cell stage parthenotes were transferred into recipients, which was different from the previous procedure in which parthenotes were cultured *in vitro* overnight for transfer. One from 4 transferred animals was pregnant and 16 fetuses were collected after 30 day of gestation. All the large foetuses had normal morphology compared to normal controls at the same developmental stage, but they were smaller. The data show for the first time that ovulated pig parthenotes can survive *in vivo* up to day 30 of gestation, which extends the previous observation (Kure-bayashi *et al,* 2000). However, *in vivo* developmental competence of pig parthenotes has not been determined.

### 5.6.5. *In vivo* developmental competence of IVM parthenotes

This experiment was designed to determine *in vivo* developmental competence of pig parthenotes, also to improve the efficiency of parthenote fetal implantation. Similar to the last experiment, the synchronisation between activated oocytes and recipient animals was obviously one of major factors influencing pregnancy rate in this experiment since the first four recipient animals that exhibited oestrus 3 days before transfer were used, but none of them became pregnant following embryo transfer. Subsequently, 11 from 13 transferred recipients (85%) were pregnant after day 2 recipients were used instead of day 3 recipients. Interestingly, day 2 or day 2.5 Meishan (equivalent to day 3 or 3.5 in this experiment) could be used as suitable recipients for transfer of pig parthenotes cultured for 2 days in a previous experiment (Kure-bayashi *et al*, 2000). Based on these conflicting results it could be suggested that recipients from different breeds could affect pregnancy rate after embryo transfer, also it would be reasoned that Meishan pigs may be more effective as recipients than Large-White as they have a relatively longer period for transferred embryos to survive. Although Large-White pig being a recipient seems to be not as effective as Meishan, the pregnancy rate of 85% on day 21 after activated oocyte transfer regardless of the use of day 3 recipients used was higher than or at least comparable to the normal pregnancy rate of 60–75% reported after surgical transfers (Cameron et al., 1989). This suggests that transfer of parthenogenetic embryos to recipient animals requires a stricter synchronisation between parthenogenetic embryos and the recipient animal if parthenotes are cultured *in vitro* for a longer period, compared to those for a short period of culture. Also, these results demonstrate that this system of production of

pig parthenotes is efficient and available for maintaining a pregnancy in pig nuclear transfer.

It is interesting that parthenogenetic embryos can survive *in vivo* for 21 days in sheep (Loi *et al*, 1998) for 30 days in cattle (Fukui *et al*, 1992) and for 29 days in pigs (Kure-bayashi *et al*., 2000). Sheep parthenotes die by day 25 (Loi *et al*., 1998). Although *in vivo* development of parthenogenetic pig embryos beyond the blastocyst stage at day12 following electrical activation and at the stage of limb-bud formation at 29 days post activation have been reported (Jolliff and Prather, 1997; Kure-bayashi et al, 2000), *in vivo* developmental competence of parthenogenetic pig embryos has not been determined. In this experiment, the results clearly show that parthenogenetic pig fetuses from *in vitro* maturation can survive *in vivo* for over 30 days and that they stop development around day 31. There are at least two pieces of direct strong evidence to verify this conclusion, (1) heart beatings in parthenote foetuses on day 31 of gestation were apparently seen on ultrasound scans and (2) morphology of the largest parthenote collected day 35 showed a slightly advanced development compared to either day 30 IVM parthenotes or day 30 fertilised controls although its body weight and crown-rump length were not beyond those in the largest day 30 parthenotes, the differences could be visible especially in ear and eyes (Figs 5.2, 5.3 and 5.4). Interestingly, parthenogenetic pig fetuses can survive *in vivo* much longer than sheep parthenotes although sheep has a longer gestation than that in pigs (148 days vs.114 days). In sheep, full implantation of fertilised embryos takes place on day 23-25 of gestation, whereas it occurs on day 25-26 of gestation in pigs. After full implantation, pig parthenotes can still develop for 4–5 days, whereas sheep parthenotes die close to the full implantation. The reason for the death of

parthenogenetic pig embryos is not fully known. In mouse, parthenogenetic embryos can develop fairly normally to the 25-somite stage, but they are always smaller than fertilised controls and even in these well-developed parthenogenetic conceptuses, the extra-embryonic tissues develop poorly (Surani, 1991; Varmuza *et al*, 1993). In addition, it has been well known that mouse parthenotes do not develop a functional placenta (Barton *et al* 1984, Kaufmann, *et al* 1977), In the mouse, precise analysis of double mutants in Igf2r and Igf2 has recently revealed effects of lack of mannose-6-phosphate binding capacity on postnatal life, but in fetal life the key role of IGF2R seems indeed to be one of lowering the levels of active IGF2. In rabbits, the parthenogenetic rabbit fetuses were of smaller size than the controls, the development of the trophoblast tissue was proportional to the development of the fetuses, but abnomalies of rabbit fetuses were observed (Ozil, 1990). In the pig parthenotes, the large day 35-parthenote fetuses were externally normal, which was likely to indicate that pig parthenogenetic fetuses may die due to impairment of extra-embryonic cell function (Latham, 1996) because gynogenotes are deficient in extra embryonic tissues and androgenotes poorly develop embryonic tissue (McGrath and Solter; 1984). It was reported that parthenogenetic pig diploids were not fit to develop until day 29-post activation because fetuses with mortal abnormalities were weeded out during gestation while the proportion of development to fetuses might also have been decreased (Kure-bayashi *et al*, 2000). However, the results here showed that the proportions of transferred cleaved oocytes developing to the postimplantation stage on day 21, day 30 and day 35 were 20% (32/160), 16.4% (37/226) and 28.6% (20/70), respectively. Of which, the proportion on day 35 was significantly higher than that on day 30 ($p<0.05$, $\chi^2$-test). In fact, this

significant difference may reflect variations among individual recipients rather than a real increase of the fetal development. Moreover, in this case, overcrowding of transferred embryos might also affect normal development of pig parthenotes because an average of 40 activated oocytes (a range from 35 to 44) were transferred into each recipient whose fetuses were collected on day 21, whereas an average of 45 (42-48) and 35 (2x35) were transferred for day 30 and day 35 fetal collections, respectively (Table 5.5). These numbers of transferred embryos were apparently higher than naturally normal ovulations (a range from 12 to 25 oocytes) in pigs. Surprisingly, the percentage of development to foetuses seems to increase with the reduction of the number of transferred embryos in this case. In the similar experiment reported, an average of 25 parthenogenetic embryos with a range from 19 to 49 cleaved pig parthenotes were transferred into each recipient, which resulted in an implantation rate of over 30% (Kure-bayashi *et al*, 2000). No doubt, embryo implantation rate is significantly influenced by the number of embryos transferred regardless of the effect of the recipients. For instance, embryo implantation rate was 64% after the transfer of 12–15 embryos and 45% after transfer of 16–20 embryos (Brüssow, 1990). The number of transferred oocytes in these experiments may be still too high and needs to be optimised. In addition, transferring cleaved parthenotes to animals seemed to improve pregnancy rate although the number of the transferred parthenotes was reduced compared to that in experiment 3 (63 vs. 85%). And an extra day culture of parthenotes seemed not to affect fetal size and body weight significantly.

In addition, abnormalities appeared in some fetuses, such as formation of cyst-like structures in heart and liver, and some visible deficiencies of the head

region. These observations are same as those reported (Kure-bayashi *et al*, 2000). The reasons for the abnormalities may reflect some damages at cell or gene level during activation and culture.

In summary, these results show that the current electrical activation protocol can yield a blastocyst rate of over 40% with either ovulated or IVM porcine oocytes. *In vivo* development of oocytes activated by this protocol has verified that this protocol can be used for pig nuclear transfer. In addition, it has been confirmed for the first time that parthenogenetic pig embryos are capable of developing *in vivo* for 30 days and that they stop development around day 31 of gestation. The combination of *in vivo* developmental competence of IVM pig parthenotes with a pregnancy rate of 85% of parthenogenetic cleaved embryos on day 21 indicates that the use of such parthenogenetic pig embryos in a supportive role in the establishment of pregnancy in recipients carrying a limited number of fertilised or cloned embryos is possible in either theory or practical. More research such as morphology, histology of pig parthenotes at different developmental stages, as well as their membrane and imprinted genes needs to be done to investigate status of pig parthenotes during development *in vivo*.

Taken all the data in this chapter together, it is concluded that this current electrical activation protocol for pig oocytes is efficient and suitable for pig nuclear transfer and production of pig parthenotes.

# Chapter 6

## General Discussion

During the studies presented in this thesis, an *in vitro* culture system and an effective electrical activation protocol for porcine oocytes have been established. I have determined for the first time the *in vitro* and *in vivo* developmental competence of porcine parthenogenetic embryos. Parthenogenetic embryos can develop *in vivo* for at least 30 days and stop developing around day 31. To compare the best *in vitro* development of electrically activated oocytes in pigs so far (Grupen *et al*, 2000), this protocol seems to be simpler and more efficient for both *in vivo*- and *in vitro* matured oocytes. Grupen *et al* (2000) applied a second set of activating pulses which consisted of two 1.5 kV/cm DC pulses of 60 μsec with an interval of 1 sec 30 min after the first set to activate *in vivo* derived oocytes, improving the blastocyst rate compared to a single treatment (51 vs. 34%). However, the same protocol used to activate *in vitro* matured oocytes resulted only in the rate of 16 % compared to 31% with a set of pulses, which indicates that two sets of such stimuli are detrimental to *in vitro* matured oocytes. In addition, from practical point of view, Grupen's protocol takes a longer time than mine as oocytes have to be cultured for 30 min after the first set of pulses, thus they have to change different environments several times during which the developmental competence of these oocytes may be affected. Presumably, multiple electrical pulses mimic the intracellular calcium oscillations of fertilised oocytes more closely than a single pulse. However, recent studies indicate that the mechanisms of downregulation in

fertilised- and parthenogenetically activated oocytes may be different (Mehlmann *et al.*, 1996; Parrington *et al.*, 1998; Brind *et al*, 2000). Therefore, this difference may reflect a limitation of such the mimic. Although multiple electrical pulses have shown to effectively induce the parthenogenetic development of mouse (Vitullo and Ozil, 1992), rabbit (Collas and Robl 1990; Ozil 1990), cattle (Collas *et al* 1993) and pig (Grupen *et al*, 1999), we have not yet known the detailed mechanism. Also, many factors that have been discussed in the previous chapters could contribute the developmental competence of activated oocytes, which make more difficult to compare different protocols. However, oocyte activation has a number of applications;

1. The electrical activation protocol can be used for activation of reconstructed porcine embryos. In fact, this protocol has been applied for porcine nuclear transfer, resulting in the birth of a healthy male piglet cloned from a fetal fibroblast cell (Fig. 6.1) (DeSousa *et al.*, 2001 accepted)

**Fig. 6.1.** A piglet cloned from a fibroblast cell

2. The electrical activation protocol can also be applied to produce parthenogenetic embryos and fetuses, which are useful for studying, imprinted genes of either maternal or paternal loci (Kono *et al* 1996; Surani *et al* 1984; Surani *et al*, 1987).

3. In addition to studying imprinted genes, parthenogenetic embryos as helpers can be co-transferred with cloned embryos to an animal in order to support cloned embryos to term. To date, this method would be an alternative for maintaining a pregnancy in pigs, which may be useful for pig nuclear transfer as current methods including co-transfer of fertilised embryos, hormonal treatment to maintain a pregnancy seem to be not efficient (Polejaeva *et al*, 2000;). The current method with which cloned piglets were successfully achieved is to transfer as many reconstructed embryos as possible into one animal so that more than 4 embryos would be expected to survive. It is relevant that more people are certainly required to do micromanipulation in order to produce more reconstructed embryos. Also, if less than 4 embryos survive, the pregnancy would not be maintained. Its limitation is apparent. During the studies, activated oocytes were co transferred with either 3 fertilised or cloned embryos to animals, 2 healthy piglets (Fig. 6.2.) (King *et al.*, 2001 submitted) and one healthy cloned piglet were achieved (Fig.6.1) Especially, one pregnancy carrying cloned embryos and parthenotes from 3 co-transferred animals developed to term, suggesting that this method could be more effective than the others current methods to maintain a pregnancy in pigs, such as co-transfer of cloned embryos with fertilised embryos, treatment of hormones (Table 6.1).

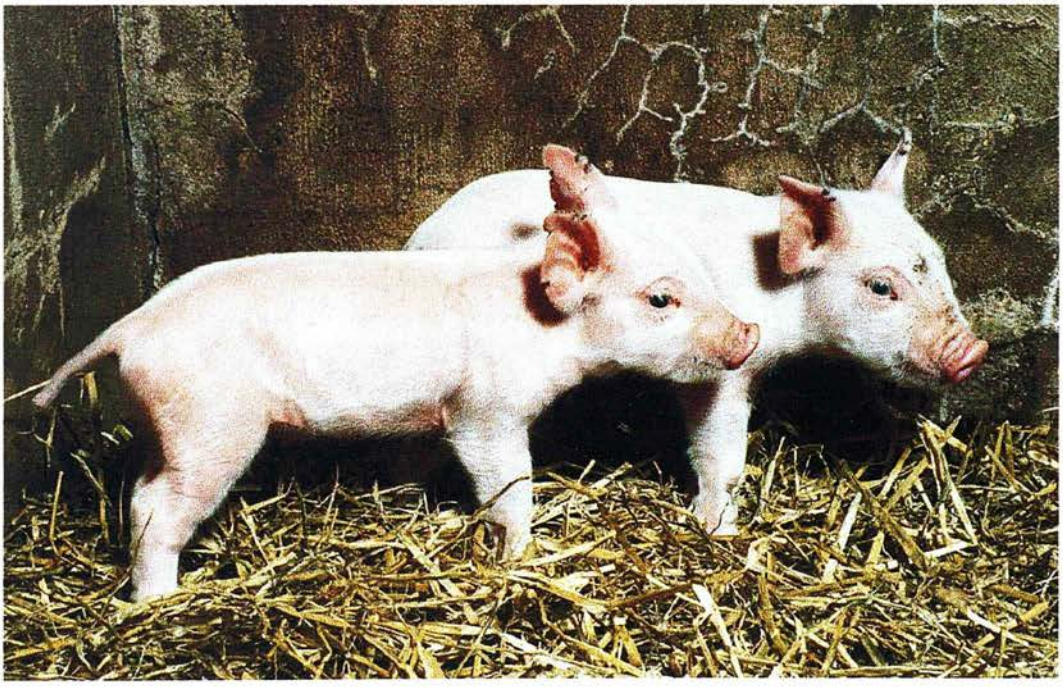
**Table 6. 1.** Number of pregnancies established and number of days maintained following co-transfer of parthenogenetic and fertilised embryos to 6 recipients. *The day at which a pregnancy was lost was deemed to be the mid point between the last ultrasound examination at which the pregnancy was still visible and the next examination at which it was not. Examinations were carried out twice weekly (King *et al.*, 2001 submitted)

**Fig.6.2.** Two piglets from three fertilised embryos co-transferred with IVM parthenotes

**Table 6. 1. Number of pregnancies established and number of days maintained following co-transfer of parthenogenetic and fertilised embryos to 6 recipients.**

| Replicate | No. of Parthenotes | No. fertile embryos | Day of pregnancy loss * |
|---|---|---|---|
| 1 | 59 | 3 | Not Pregnant |
| | 59 | 3 | Two live piglets at term |
| 2 | 60 | 3 | 72* |
| | 60 | 3 | 45* |
| 3 | 60 | 3 | 60* |
| | 58 | 3 | Not Pregnant |

**Fig.6.2.**

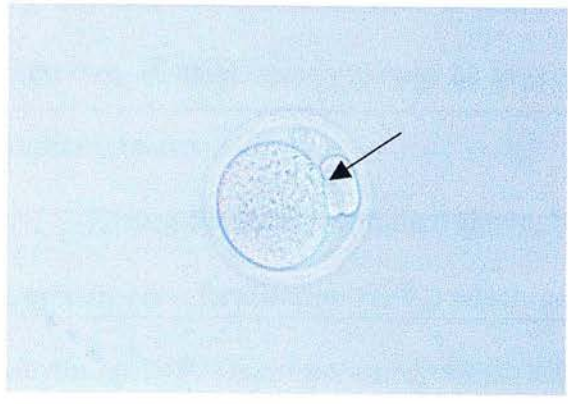


4. The activation protocol could also be applied for chemical enucleation in porcine oocytes. It is known that in animal nuclear transfer procedures oocytes used as recipient cytoplasts must be enucleated mechanically by micromanipulation, but more cytoplasm would be removed during this process, which could affect donor nuclear reprogramming. However, a new technique to enucleate oocytes with chemicals such as microtubule inhibitors has recently been developed, namely mature oocytes can be artificially activated and treated with a microtubule inhibitor for a short time, the metaphase plate would be able to extrude with the second polar body together. So only a small amount of cytoplasm surrounding the metaphase plate extrudes, which is much easily removed by manipulation. This technique is called **chemical enucleation**. Chemical enucleation has successfully been used for mouse nuclear transfer; it seemed to be more effective than the conventional method of nuclear transfer (Kishikawa *et al*, 1999). Clearly, this method requires oocyte activation. Therefore, an effective activation protocol would logically improve the efficiency of chemical enucleation. In addition, enucleated part in porcine oocytes were smaller than that in mouse oocytes, which indicates that chemical enucleation in porcine oocytes may have an advantage in developmental potential compared to that in mouse nuclear transfer (Fig.6.3. A, B, C and D). This technique is much simpler compared to the conventional, mechanical enucleation; in addition to the advantages described above the big advantage is that a large number of oocytes could be enucleated chemically at the same time. Although I have successfully enucleated porcine oocytes chemically with this activation protocol, more research needs to be done. It would be predicted that chemical enucleation might

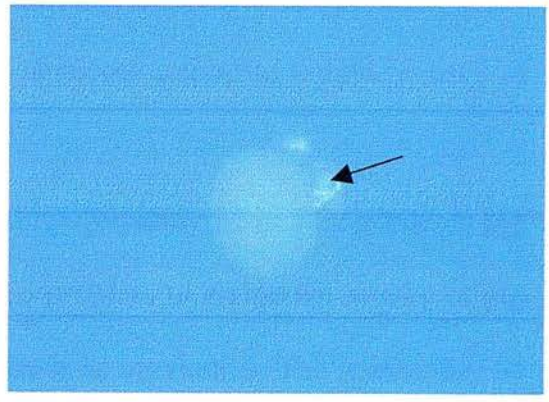
provide a simple, effective method for nuclear transfer since enucleation seems to be the big hurdle to be overcome or avoided in nuclear transfer procedures.

In addition, except for the studies presented in this thesis, some preliminary experiments with other activation agents such as sperm protein factors, ethanol and Strontium ($Sr^{2+}$) were also carried out. All these agents have been proven to be able to activate porcine oocytes and activated oocytes can develop to the blastocyst stage. Of which, blastocyst formation after activation by either sperm protein factor or $Sr^{2+}$ are reported here for the first time, however, the blastocyst rate was still low, below 10 % in both cases. Only 4 blastocysts were observed from 93 sperm protein factor injected oocytes, but technical problem of microinjection certainly contributed to this inefficiency. However, it is suggested that sperm factor could activate pig oocytes and cause cleavage and development of the oocytes to the blastocyst stage with a range of nuclei from 29 to 42 in the blastocysts. Clearly, more experiments with these agents need to be carried out to optimise activation conditions in order to improve their efficiencies. For example, $Sr^{2+}$ can effectively activate mouse oocytes (Wakayama *et al.*, 1998), but the same concentration of $Sr^{2+}$ as in activating mouse oocytes seems to be not suitable for activating porcine oocytes, porcine oocytes seem to require higher concentration of $Sr^{2+}$. However, a high concentration of $Sr^{2+}$ will increase osmolarity in the medium, leading to damage or kill pig oocytes. An alternative may be to apply multiple treatments with a low concentration of $Sr^{2+}$ or ethanol to activate oocytes instead of high concentration of the agents

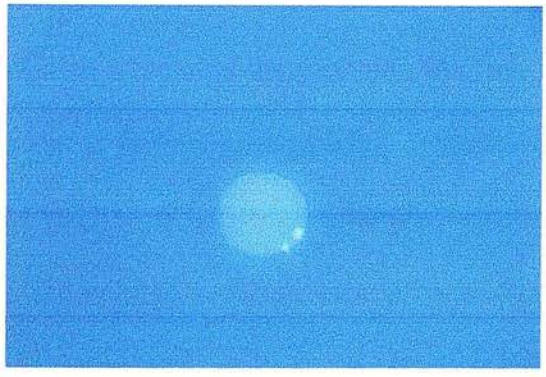
**Fig.6.3.** A chemically enucleated mouse oocytes under lights (Magnifications: X 400). The arrow points the enucleated nucleus (**A**); the chemically enucleated mouse oocytes.stained by Hoechst 33342 under UV lights. The arrow points the enucleated nucleus (Magnifications: X400) (**B**). A chemically enucleated pig oocyte stained with Hoechst 33342 under UV lights (Magnifications: X 200) (**C**). The chemically enucleated pig oocyte under lights. (**D**)



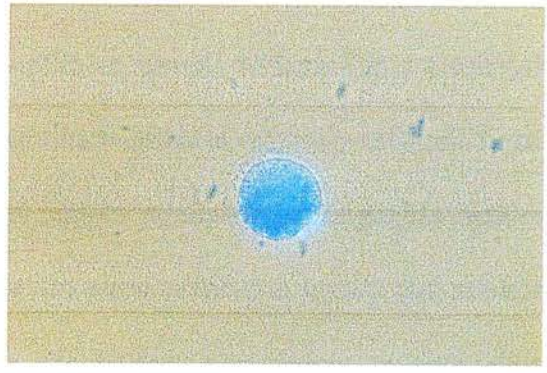
A.



B.



C.



D.

Recently, ionomycin-a chemical agent successfully activating oocytes in sheep (Loi *et al*, 1998) and pigs (Betthauser *et al*., 2000) has been reported. Compared with electrical activation, chemical activation is much easier in practice and does not require any expansive machine as well as many oocytes can be treated at the same time. Therefore, if its efficiency would be improved, chemical activation would be used in nuclear transfer.

Except for these mentioned above, because polyspermy frequently occurs in pig oocyte *in vitro* fertilisation (IVF), which causes a problem to assess the quality of pig oocytes by IVF. Therefore, using oocyte activation is an alternative. The results may be more reliable and practical compared to IVF.

In spite of the improvement of pig oocyte activation by these studies, the efficiency of pig oocyte activation still needs to be improved. Also, the related techniques need to be optimised such as maturation system, culture system in order to improve the quality of oocytes. In addition, problems associated with oocyte age need to be overcome in oocyte activation especially in nuclear transfer. There would be two ways to overcome the problem; the first is to optimise stimuli with multiple electrical pulses at different oocyte ages. It is relevant that this method is certainly difficult since the quality of oocytes, maturation medium and culture conditions–changeable factors would involve in the efficiency; the second is to use some activation agents that are not age–dependent, such as sperm protein factor. Therefore, two points in activating reconstructed embryos should seriously be considered, (1) how efficient the activation method is in activating reconstructed embryos; (2) when the activation should take

place. Although the efficiency of nuclear transfer is still very low, it would be improved rapidly with the improvements of various related techniques including oocyte activation.

Additionally, some important progress on the mechanism of fertilisation has been made recently. For instance, in the mouse Type I inositol trisphosphate receptor ($InsP_3$ R) protein increased during oocyte maturation and, in addition, within 8 h of fertilisation underwent a dramatic decrease. During development to the blastocyst the level of type I $InsP_3$ R protein did not return to prefertilisation levels and Types II and III remained below the detection limit. The decrease in $InsP_3$ R after fertilisation was correlated with a decrease in the sensitivity of $InsP_3$ – induced $Ca^{2+}$ release. These data show that the expression of $InsP_3$ R mRNA is developmentally regulated, that $Ca^{2+}$ signalling at fertilisation is mediated exclusively through the type I $InsP_3$ R, and that the $InsP_3$ R is down - regulated after fertilisation (Mehlmann *et al.*, 1996; Parrington *et al.*, 1998). However, Brind *et al*, (2000) find that neither egg activation nor $Ca^{2+}$ transients are necessary or sufficient for the stimulation of $InsP_3R$ down regulation. This conclusion is supported by several experiments, (1) parthenogenetic activation fails to stimulate down regulation; (2) down regulation persists when fertilisation induced $Ca^{2+}$ transients and egg activation are inhibited using BAPTA (3) down regulation can be induced in immature oocytes that did not undergo egg activation. $InsP_3R$ down regulation was inhibited by the cysteine protease inhibitor ALLN but MG123 and lactatcystin were not effective. Moreover, maturing oocytes were injected with adenophstin A and produced MII egg depleted of $InsP_3Rs$, in which sperm – induced

$Ca^{2+}$ signalling was inhibited. These interesting findings indicate that the mechanisms of down regulation in fertilised- and parthenogenetically activated oocytes are different. It is known that cell-cycle- dependent changes in the ability to release $Ca^{2+}$ are one mechanism that leads to the inhibition of $Ca^{2+}$ transients after fertilisation, but we do not know whether this mechanism is also available in parthenogenetic activation, too. Based on these studies, it can be concluded that the mechanism of parthenogenetic activation in mammals remains highly nuclear. However, it is interesting to know whether cell cycle – dependent changes would be related to the efficiency of parthenogenetic activation. If yes, the current activation protocols for animal cloning should be changeable according the use of different species and different sources of tissue as donor cells that may have a different cell cycle. Undoubtedly, more research on the mechanism of parthenogenetic activation should be done in order to contribute to its basic understanding and to improve the current activation protocols for animal cloning.

(1998). Molecularly cloned mammlian glucosamine-6 phosphate deaminase localizes to the transporting epithelium and lacks oscillin activity. FASEB J *12*, 91-99.

Abassi,Y.A. and Foltz,K.R. (1994). Tyrosine phosphorylation of the egg receptor for sperm at fertilization. Dev Biol *164*, 430-443.

Abeydeera,L.R., Wang,W.H., Cantley,T.C., Rieke,A., Prather,R.S., and Day,B.N. (1998). Presence of epidermal growth factor during in vitro maturation of pig oocytes and embryo culture can modulate blastocyst development after in vitro fertilization. Mol Reprod Dev *51*, 395-401.

Aoyagi,Y. (1992). Artificial activation of bovine oocytes matured in vitro by electric shock or exposure to ionophore A23187. Theriogenology *37*, 180-188.

Baguisi,A., Behboodi,E., Melican,D.T., Pollock,J.S., Destrempes,M.M., Cammuso,C., Williams,J.L., Nims,S.D., Porter,C.A., Midura,P., Palacios,M.J., Ayres,S.L., Denniston,R.S., Hayes,M.L., Ziomek,C.A., Meade,H.M., Godke,R.A., Gavin,W.G., Overstrom,E.W., and Echelard,Y. (1999). Production of goats by somatic cell nuclear transfer. Nat Biotechnol *17*, 456-461.

Baker,P.F. and Knight D.E. (1978). A high voltage technique for gaining rapid access to the interior of secretory cells. J Physiol *284*, 30.

Baker,P.F. and Knight D.E. (1978). Influence of anions on exocytosis in leaky bovine adrenal medullary cells. J Physiol *296*, 106.

Barton,S.C., Surani,M.A., and Norris,M.L. (1984). Role of paternal and maternal genomes in mouse development. Nature *311*, 374-376.

Ben-Yosef,D., Oron,Y., and Shalgi,R. (1993). Prolonged, repetitive calcium transients in rat oocytes fertilized in vitro and in vivo. FEBS Lett *331*, 239-242.

Ben-Yosef,D., Oron,Y., and Shalgi,R. (1996). Intracellular pH of rat eggs is not affected by fertilization and the resulting calcium oscillations. Biol Reprod *55*, 461-468.

Ben-Yosef,D., Talmor,A., Shwartz,L., Granot,Y., and Shalgi,R. (1998). Tyrosyl-phosphorylated proteins are involved in regulation of meiosis in the rat egg. Mol Reprod Dev *49*, 176-185.

Ben-Yosef,D. and Shalgi,R. (1998). Early ionic events in activation of the mammalian egg. Rev Reprod *3*, 96-103.

Berrie,C.P., Cuthbertson,K.S., Parrington,J., Lai,F.A., and Swann,K. (1996). A cytosolic sperm factor triggers calcium oscillations in rat hepatocytes. Biochem J *313 ( Pt 2)*, 369-372.

Betthauser,J., Forsberg,E., Augenstein,M., Childs,L., Eilertsen,K., Enos,J., Forsythe,T., Golueke,P., Jurgella,G., Koppang,R., Lesmeister,T., Mallon,K., Mell,G., Misica,P., Pace,M., Pfister-Genskow,M., Strelchenko,N., Voelker,G., Watt,S., Thompson,S., and Bishop,M. (2000). Production of cloned pigs from in vitro systems. Nat Biotechnol *18*, 1055-1059.

Bjornson,C.R., Rietze,R.L., Reynolds,B.A., Magli,M.C., and Vescovi,A.L. (1999). Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. Science *283*, 534-537.

Bondioli,K.R. (1993). Nuclear transfer in cattle. Mol Reprod Dev *36*, 274-275.

Bos-Mikich,A., Whittingham,D.G., and Jones,K.T. (1997). Meiotic and mitotic Ca2+ oscillations affect cell composition in resulting blastocysts. Dev Biol *182*, 172-179.

Bromhall,J.D. (1975). Nuclear transplantation in the rabbit egg. Nature *258*, 719-722.

Brussow,K.P. (1990). Results of embryo transfer into the oviduct or uterus in pigs. Monatshefte fur Veterinarmedizin *45*, 562-565.

Brustle,O., Choudhary,K., Karram,K., Huttner,A., Murray,K., Dubois-Dalcq,M., and McKay,R.D. (1998). Chimeric brains generated by intraventricular transplantation of fetal human brain cells into embryonic rats. Nat Biotechnol *16*, 1040-1044.

Calvin,H.I., Grosshans,K., and Blake,E.T. (1986). Estimation and manipulation of gluthione levels in prepubertal mouse ovaries and ova: relevance to sperm nucleus transformation in the fertilized egg. Gamete Res *14*, 265-275.

Cameron,R.D.A., Durack,M., Forgarty,R., Putra,D.K.H., and McVeigh,J. (1989). Practical experience with commercial embryo transfer in pigs. Austr Veterin *66*, 314-318.

Campbell,K.H., McWhir,J., Ritchie,W.A., and Wilmut,I. (1996). Sheep cloned by nuclear transfer from a cultured cell line. Nature *380*, 64-66.

Campbell,K.H. and Wilmut,I. (1998). Nuclear Transfer. In Animal Breeding Technology for the 21st Century, A.J.Clark, ed. (Amsterdam: Harwood academic), pp. 47-62.

Carroll,J. and Swann,K. (1992). Spontaneous cytosolic calcium oscillations driven by inositol trisphosphate occur during in vitro maturation of mouse oocytes. J Biol Chem *267*, 11196-11201.

Carroll,J., Jones,K.T., and Whittingham,D.G. (1996). Ca2+ release and the development of Ca2+ release mechanisms during oocyte maturation: a prelude to fertilization. Rev Reprod *1*, 137-143.

Cha,S.K., Kim,N.H., Lee,S.M., Baik,C.S., Lee,H.T., and Chung,K.S. (1997). Effect of cytochalasin B and cycloheximide on the activation rate, chromosome constituent and in vitro development of porcine oocytes following parthenogenetic stimulation. Reprod Fertil Dev *9*, 441-446.

Chang D.C (1992). Guide to elecroporation and electrofusion. Academic Press).

Chang,D.C., Saunders,J.A., Chassy,B.M., and Sowers,A.E. (1992). Overview of electroporation and electrofusion. In Guide to Electroporation and Electrofusion, D.C.Chang, J.A.Saunders, B.M.Chassy, and A.E.Sowers, eds. Acadeni Press), pp. 1-6.

Cheng W.T.K, Moor,R.M., and and Polge.C (1986). In vitro fertilisation of pig and sheep oocytes matured in vivo and in vitro. Theriogenology *25*, 146.

Cibelli,J.B., Stice,S.L., Golueke,P.J., Kane,J.J., Jerry,J., Blackwell,C., Ponce,d.L.F., and Robl,J.M. (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science *280*, 1256-1258.

Clarke,H.J. and Masui,Y. (1983). The induction of reversible and irreversible chromosome decondensation by protein synthesis inhibition during meiotic maturation of mouse oocytes. Dev Biol *97*, 291-301.

Collas,P., Balise,J.J., Hofoman,G.A., and Robl,J.M. (1989). Electrical activation of mouse oocytes. Theriogenology *32*, 835-844.

Collas,P. and Robl,J.M. (1990). Factors affecting the efficiency of nuclear transplantation in the rabbit embryo. Biol Reprod *43*, 877-884.

Collas,P. and Barnes,F.L. (1992). Parthenogenetic development of bovine oocytes. Biol Reprod *46*, 65.

Collas,P., Fissore,R., Robl,J.M., Sullivan,E.J., and Barnes,F.L. (1993). Electrically induced calcium elevation, activation, and parthenogenetic development of bovine oocytes. Mol Reprod Dev *34*, 212-223.

Colman,A. (1999). Dolly, Polly and other 'ollys': likely impact of cloning technology on biomedical uses of livestock. Genet Anal *15*, 167-173.

Cuthbertson,K.S., Whittingham,D.G., and Cobbold,P.H. (1981). Free Ca2+ increases in exponential phases during mouse oocyte activation. Nature *294*, 754-757.

DeChiara,T.M., Robertson,E.J., and Efstratiadis,A. (1991). Parental imprinting of the mouse insulin-like growth factor II gene. Cell *64*, 849-859.

Didoion,B.A., Martin,M.J., and Market,C.L. (1990). Parthenogenetic activation of mouse and pig oocytes matured in vitro. Theriogenology *49*, 43-52.

Ding,J. and Foxcroft,G.R. (1994). Epidermal growth factor enhances oocyte maturation in pigs. Mol Reprod Dev *39*, 30-40.

Dobrinsky, J. R Purrsel V. G. Long C. R. Johnson L. A. Brith of piglets after transfer of embryos cryopreserved by cytoskeletal stabilization and vitrification. Biology of Reproduction 62, 564-570. 2001.
Ref Type: Generic

Edwards,R.G., Steptoe,P.C., and Purdy,J.M. (1980). Establishing full-term human pregnancies using cleaving embryos grown in vitro. Br J Obstet Gynaecol *87*, 737-756.

Evans,M.J., Gurer,C., Loike,J.D., Wilmut,I., Schnieke,A.E., and Schon,E.A. (1999). Mitochondrial DNA genotypes in nuclear transfer-derived cloned sheep. Nat Genet *23*, 90-93.

Farkas DL (1989). External electric field- induced transmembrane potential in biological systems: Features, Effects and optical Monitoring. In Electroporation and electrofusion in cell biology, S.A.a.J.C.Neumann E, ed. (New york and London: Plenum Press Inc.), pp. 409-431.

Feil,R., Khosla,S., Cappai,P., and Loi,P. (1998). Genomic imprinting in ruminants: allele-specific gene expression in parthenogenetic sheep. Mamm Genome *9*, 831-834.

Fissore,R.A. and Robl,J.M. (1992). Intracellular Ca2+ response of rabbit oocytes to electrical stimulation. Mol Reprod Dev *32*, 9-16.

Fissore,R.A., Gordo,A.C., and Wu,H. (1998). Activation of development in mammals: is there a role for a sperm cytosolic factor? Theriogenology *49*, 43-52.

Fissore,R.A., Reis,M.M., and Palermo,G.D. (1999). Isolation of the Ca2+ releasing component(s) of mammalian sperm extracts: the search continues. Mol Hum Reprod *5*, 189-192.

Flax,J.D., Aurora,S., Yang,C., Simonin,C., Wills,A.M., Billinghurst,L.L., Jendoubi,M., Sidman,R.L., Wolfe,J.H., Kim,S.U., and Snyder,E.Y. (1998).

Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. Nat Biotechnol *16*, 1033-1039.

Foltz,K.R. (1995). Sperm-binding proteins. Int Rev Cytol *163*, 249-303.

Fujiwara,T., Nakada,K., Shirakawa,H., and Miyazaki,S. (1993). Development of inositol trisphosphate-induced calcium release mechanism during maturation of hamster oocytes. Dev Biol *156*, 69-79.

Fukui,Y., Sawai,K., Furudate,M., Sato,N., Iwazumi,Y., and Ohsaki,K. (1992). Parthenogenetic development of bovine oocytes treated with ethanol and cytochalasin B after in vitro maturation. Mol Reprod Dev *33*, 357-362.

Funahashi,H. and Day,B.N. (1993). Effects of the duration of exposure to hormone supplements on cytoplasmic maturation of pig oocytes in vitro. J Reprod Fertil *98*, 179-185.

Funahashi,H., Cantley,T., and Day,B.N. (1994). Different hormonal requirements of pig oocyte-cumulus complexes during maturation in vitro. J Reprod Fertil *101*, 159-165.

Funahashi,H., Cantley,T.C., Stumpf,T.T., Terlouw,S.L., and Day,B.N. (1994). Use of low-salt culture medium for in vitro maturation of porcine oocytes is associated with elevated oocyte glutathione levels and enhanced male pronuclear formation after in vitro fertilization. Biol Reprod *51*, 633-639.

Funahashi,H., Kim,N.H., Stumpf,T.T., Cantley,T.C., and Day,B.N. (1996). Presence of organic osmolytes in maturation medium enhances cytoplasmic maturation of porcine oocytes. Biol Reprod *54*, 1412-1419.

Furuichi,T., Yoshikawa,S., Miyawaki,A., Wada,K., Maeda,N., and Mikoshiba,K. (1989). Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P400. Nature *342*, 32-38.

Gauger,B. and Bentrup,F.W. (1979). A study of dielectric membrane breakdown in the Fucus egg. J Membr Biol *48*, 249-264.

Gilbert,S.H., Perry,K., and Fay,F.S. (1994). Mediation of chemoattractant-induced changes in [Ca2+]i and cell shape, polarity, and locomotion by InsP3, DAG, and protein kinase C in newt eosinophils. J Cell Biol *127*, 489-503.

Grupen,C.G., Nagashima,H., and Nottle,M.B. (1997). Role of epidermal growth factor and insulin-like growth factor-I on porcine oocyte maturation and embryonic development in vitro. Reprod Fertil Dev *9*, 571-575.

Grupen,C.G., Verma,P.J., Du,Z.T., McIlfatrick,S.M., Ashman,R.J., and Nottle,M.B. (1999). Activation of in vivo- and in vitro-derived porcine oocytes by using multiple electrical pulses. Reprod Fertil Dev *11*, 457-462.

Hagen,D.R., Prather,R.S., and First,N.L. (1991). Response of porcine oocytes to electrical and chemical activation during maturation in vitro. Mol Reprod Dev *28*, 70-73.

Hall,J.G. (1990). Genomic imprinting: review and relevance to human diseases. Am J Hum Genet *46*, 857-873.

Hirao,Y., Nagai,T., Kubo,M., miyano,T., Miyake,M., and Kato,S. (1994). In vitro growth and maturation of pig oocytes. J. Reprod. Fertil. *100*, 333-339.

Hofmann,G. (1989). Cells in Electric Fields : Physical and Practical Electronic Aspects of Electro Cell Fusion and Electroporation. Chapter 26 in Electroporation and Electrofusion in Cell Biology Edited by Neumann,E Sowers AE and Jordan CA.

Homa,S.T. and Swann,K. (1994). A cytosolic sperm factor triggers calcium oscillations and membrane hyperpolarizations in human oocytes. Hum Reprod *9*, 2356-2361.

Illera,M.J., Lorenzo,P.L., Illera,J.C., and Petters,R.M. (1998). Developmental competence of immature pig oocytes under the influence of EGF, IGF-I, follicular fluid and gonadotropins during IVM-IVF processes. Int J Dev Biol *42*, 1169-1172.

Illmensee,K. and Hoppe,P.C. (1981). Nuclear transplantation in Mus musculus: developmental potential of nuclei from preimplantation embryos. Cell *23*, 9-18.

Ilyin,V. and Parker,I. (1992). Effects of alcohols on responses evoked by inositol trisphosphate in Xenopus oocytes. J Physiol *448*, 339-354.

Jaffe,L.F. (1985). The role of calcium explosions, waves, and pulses in activating eggs. In Biology of Fertilization, C.B.Metz and A.Monroy, eds. (Orlando, FL: Academic Press), pp. 127-165.

John Parrington,S.B.H.D.S.R.G.F.A.L.R.W.a.J.C. (1998). Expression of Inositol 1,4,5-trisphosphate Receptors in Mouse Oocytes and Early Embryos: the Type I Isoform Is Upregulated in Oocytes and Downregulated after Fertilization. Developmental Biology *203*, 451-461.

Jolliff,W.J. and Prather,R.S. (1997). Parthenogenic development of in vitro-matured, in vivo-cultured porcine oocytes beyond blastocyst. Biol Reprod *56*, 544-548.

Jones KT,S.C. and Cannell MB (1998). The passage of Ca2+ and flurescent markers between the sperm and egg after fusion in the mouse. Development *125*, 4627-4635.

Jung,Y.G., Sakata,E.S., Lee,E.S., and Fukui,Y. (1998). Amino acid metabolism of bovine blastocysts derived from parthenogenetically activated or in vitro fertilized oocytes. Reprod Fertil Dev *10*, 279-287.

Ka,H.H., Sawai,K., Wang,W.H., Im,K.S., and Niwa,K. (1997). Amino acids in maturation medium and presence of cumulus cells at fertilization promote male pronuclear formation in porcine oocytes matured and penetrated in vitro. Biol Reprod *57*, 1478-1483.

Karl Swann and John Parrington (1999). Mechanism of Ca2+ release at fertilization in mammals. Journal of experimental Zoology (Mol Dev Evol) *285*, 267-275.

Kato,Y., Tani,T., Sotomaru,Y., Kurokawa,K., Kato,J., Doguchi,H., Yasue,H., and Tsunoda,Y. (1998). Eight calves cloned from somatic cells of a single adult. Science *282*, 2095-2098.

Kaufman,M.H. and Sachs,L. (1975). The early development of haploid and aneuploid parthenogenetic embryos. J Embryol Exp Morphol *34*, 645-655.

Kaufman,M.H., Barton,S.C., and Surani,M.A. (1977). Normal postimplantation development of mouse parthenogenetic embryos to the forelimb bud stage. Nature *265*, 53-55.

Kikuchi,K., Izaike,Y., Noguchi,J., Furukawa,T., Daen,F.P., Naito,K., and Toyoda,Y. (1995). Decrease of histone H1 kinase activity in relation to parthenogenetic activation of pig follicular oocytes matured and aged in vitro. J Reprod Fertil *105*, 325-330.

Kim,N.H., Moon,S.J., Prather,R.S., and Day,B.N. (1996). Cytoskeletal alteration in aged porcine oocytes and parthenogenesis. Mol Reprod Dev *43*, 513-518.

Kim,N.H., Uhm,S.J., Ju,J.Y., Lee,H.T., and Chung,K.S. (1997). Blastocoele formation and cell allocation to the inner cell mass and trophectoderm in haploid and diploid pig parthenotes developing in vitro. Zygote *5*, 365-370.

Kinosita,K.Jr.a.T.T.Y. (1978). Survival of sucrose-loaded erythrocytes in circulation. Nature *272*, 258-260.

Kinosita,K.Jr.a.T.T.Y. (1979). Voltages-induced conductance by electric impulses in vesicular membrane. Biochim. Biophys. Acta *554*, 479-494.

Kishikawa,H., Wakayama,T., and Yanagimachi,R. (1999). Comparison of oocyte-activating agents for mouse cloning. Cloning *1*, 153-160.

Kline,D. and Kline,J.T. (1992). Thapsigargin activates a calcium influx pathway in the unfertilized mouse egg and suppresses repetitive calcium transients in the fertilized egg. J Biol Chem *267*, 17624-17630.

Kline,J.T. and Kline,D. (1994). Regulation of intracellular calcium in the mouse egg: evidence for inositol trisphosphate-induced calcium release, but not calcium-induced calcium release. Biol Reprod *50*, 193-203.

Knott,J.G., Kasinathan,P., Wu,H., Spell,A., Fissore,R.A., and Robl,J.M. (2001). Effect of porcine sperm factor on intracellular calcium and activation of bovine oocytes and development of nuclear transfer embryos. Theriogenology *55*, 455.

Kono,T., Iwasaki,S., and Nakahara,T. (1989). Parthenogenetic activation by electric stimulus of bovine oocytes matured in vitro. Theriogenology *33*, 569-576.

Kono,T., Carroll,J., Swann,K., and Whittingham,D.G. (1995). Nuclei from fertilized mouse embryos have calcium-releasing activity. Development *121*, 1123-1128.

Kono,T., Jones,K.T., Bos-Mikich,A., Whittingham,D.G., and Carroll,J. (1996). A cell cycle-associated change in Ca2+ releasing activity leads to the generation of Ca2+ transients in mouse embryos during the first mitotic division. J Cell Biol *132*, 915-923.

Koo,D.B., Kang,Y.K., Choi,Y.H., Park,J.S., Han,S.K., Park,I.Y., Kim,S.U., Lee,K.K., Son,D.S., Chang,W.K., and Han,Y.M. (2000). In vitro development of reconstructed porcine oocytes after somatic cell nuclear transfer. Biol Reprod *63*, 986-992.

Kotzot,D., Schmitt,S., Bernasconi,F., Robinson,W.P., Lurie,I.W., Ilyina,H., Mehes,K., Hamel,B.C., Otten,B.J., and Hergersberg,M. (1995). Uniparental disomy 7 in Silver-Russell syndrome and primordial growth retardation. Hum Mol Genet *4*, 583-587.

Kubota,C., Yamakuchi,H., Todoroki,J., Mizoshita,K., Tabara,N., Barber,M., and Yang,X. (2000). Six cloned calves produced from adult fibroblast cells after long-term culture. Proc Natl Acad Sci U S A *97*, 990-995.

Kurasawa,S., Schultz,R.M., and Kopf,G.S. (1989). Egg-induced modifications of the zona pellucida of mouse eggs: effects of microinjected inositol 1,4,5-trisphosphate. Dev Biol *133*, 295-304.

Kure-bayashi,S., Miyake,M., katayama,M., miyano,T., and kato,S. (1996). Development of porcine blastocysts from in vitro-matured and activated haploid and diploid oocytes. Theriogenology *46*, 1027-1036.

Kure-bayashi,S., Miyake,M., Okada,K., and Kato,S. (2000). Successful implantation of in vitro-matured, electro-activated oocytes in the pig. Theriogenology *53*, 1105-1119.

Kwon,O.Y. and Kono,T. (1996). Production of identical sextuplet mice by transferring metaphase nuclei from four-cell embryos. Proc Natl Acad Sci U S A *93*, 13010-13013.

Lai,F.A., Anderson,K., Rousseau,E., Liu,Q.Y., and Meissner,G. (1988). Evidence for a Ca2+ channel within the ryanodine receptor complex from cardiac sarcoplasmic reticulum. Biochem Biophys Res Commun *151*, 441-449.

Lanza,R.P., Cibelli,J.B., Blackwell,C., Cristofalo,V.J., Francis,M.K., Baerlocher,G.M., Mak,J., Schertzer,M., Chavez,E.A., Sawyer,N., Lansdorp,P.M., and West,M.D. (2000). Extension of cell life-span and telomere length in animals cloned from senescent somatic cells. Science *288*, 665-669.

Latham,K.E. (1996). X chromosome imprinting and inactivation in the early mammalian embryo. Trends Genet *12*, 134-138.

Lau,M.M., Stewart,C.E., Liu,Z., Bhatt,H., Rotwein,P., and Stewart,C.L. (1994). Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. Genes Dev *8*, 2953-2963.

Lawrence,Y., Whitaker,M., and Swann,K. (1997). Sperm-egg fusion is the prelude to the initial Ca2+ increase at fertilization in the mouse. Development *124*, 233-241.

Leal,C.L. and Liu,L. (1998). Differential effects of kinase inhibitor and electrical stimulus on activation and histone H1 kinase activity in pig oocytes. Anim Reprod Sci *52*, 51-61.

Li,G., Tan,J., Sun,Q., Meng,Q., Yue,K., Sun,X., Li,Z., Wang,H., and Xu,L. (2000). Cloned piglets born after nuclear transplantation of embryonic blastomeres into porcine oocytes matured in vivo. Cloning *2*, 45-52.

Liu,L. and Moor,R.M. (1997). Factors affecting electrical activation of porcine oocyte matured in vitro. Anim Reprod Sci *48*, 67-80.

Loi,P., Ledda,S., Fulka,J.J., Cappai,P., and Moor,R.M. (1998). Development of parthenogenetic and cloned ovine embryos: effect of activation protocols. Biol Reprod *58*, 1177-1187.

Machaty and Prather and R.S. (1998). Characteristics of thimerosal-induced activation of pig oocytes. Theriogenology *49*, 156.

Machaty,Z., Mayes,M.A., and Prather,R.S. (1995). Parthenogenetic activation of porcine oocytes with guanosine-5'-O-(3'-thiotriphosphate). Biol Reprod *52*, 753-758.

Machaty,Z. and Prather,R.S. (1998). Strategies for activating nuclear transfer oocytes. Reprod Fertil Dev *10*, 599-613.

Machaty,Z., Wang,W.H., Day,B.N., and Prather,R.S. (1999). Calcium release and subsequent development induced by modification of sulfhydryl groups in porcine oocytes. Biol Reprod *60*, 1384-1391.

Mattioli,M., Galeati,G., Bacci,M.L., and Seren,E. (1988). Follicular factors influence oocyte fertilizability by modulating the intercellular cooperation between cumulus cells and oocyte. Gamete Res *21*, 223-232.

Mattioli,M., Bacci,M.L., Galeati,G., and Seren,E. (1991). Effect of LH and FSH on the maturation of pig oocytes in vitro. Theriogenology *36*, 95-105.

Mayes,M.A., Stogsdill,P.L., and Prather,R.S. (1995). Parthenogenic activation of pig oocytes by protein kinase inhibition. Biol Reprod *53*, 270-275.

McCreath,K.J., Howcroft,J., Campbell,K.H., Colman,A., Schnieke,A.E., and Kind,A.J. (2000). Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. Nature *405*, 1066-1069.

McGaughey,R.W. (1985). Amino acids can induce nondisjunction during maturation of mammalian oocytes in vitro. J Exp Zool *234*, 161-165.

McGrath,J. and Solter,D. (1984). Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell *37*, 179-183.

Mehlmann LM,C.G.R.S.J.L. (1998). SH2 domain- mediated activation of phospholipase C gamma is required to intitiate Ca2+ release at fertilization of mouse eggs. Developmental Biology *203*, 221-232.

Mehlmann LM,C.A.C.G.J.L. (2001). Evidence that phospholipase C from the the sperm is not responsible initiating Ca 2+ release at fertilization in mouse eggs. Developmental Biology *236*, 492-501.

Mehlmann,L.M., Terasaki,M., Jaffe,L.A., and Kline,D. (1995). Reorganization of the endoplasmic reticulum during meiotic maturation of the mouse oocyte. Dev Biol *170*, 607-615.

MehlmannLM,M.K.a.K.D. (1996). Redistribution and Increase in Cortical Inositol 1, 4, 5-Trisphosphate Receptors after Meiotic Maturation of the Mouse Oocyte. Developmental Biology *180*, 489-498.

Meinecke,B. and Meinecke,T.S. (1979). Effects of gonadotropins on oocyte maturation and progesterone production by porcine ovarian follicles cultured in vitro. Theriogenology *11*, 351-365.

Minamihashi,A., Watson,A.J., Watson,P.H., Church,R.B., and schultz,G.A. (1993). Bovine parthenogenetic blastocysts following in vitro maturation and oocyte activation with ethanol. Theriogenology *40*, 63-76.

Miyazaki,S. (1988). Inositol 1,4,5-trisphosphate-induced calcium release and guanine nucleotide-binding protein-mediated periodic calcium rises in golden hamster eggs. J Cell Biol *106*, 345-353.

Miyazaki,S., Shirakawa,H., Nakada,K., and Honda,Y. (1993). Essential role of the inositol 1,4,5-trisphosphate receptor/Ca2+ release channel in Ca2+ waves and Ca2+ oscillations at fertilization of mammalian eggs. Dev Biol *158*, 62-78.

Moor,R.M.C.I.M. (1985). Temperature-induced abnormalities in sheep oocytes during maturation. J Reprod Fertil *75*, 467-473.

Moore,G.D., Kopf,G.S., and Schultz,R.M. (1993). Complete mouse egg activation in the absence of sperm by stimulation of an exogenous G protein-coupled receptor. Dev Biol *159*, 669-678.

Moore,G.D., Ayabe,T., Visconti,P.E., Schultz,R.M., and Kopf,G.S. (1994). Roles of heterotrimeric and monomeric G proteins in sperm-induced activation of mouse eggs. Development *120*, 3313-3323.

Motlik,J., Crozet,N., and Fulka,J. (1984). Meiotic competence in vitro of pig oocytes isolated from early antral follicles. J Reprod Fertil *72*, 323-328.

Motlik,J., Fulka,J., and Flechon,J.E. (1986). Changes in intercellular coupling between pig oocytes and cumulus cells during maturation in vivo and in vitro. J Reprod Fertil *76*, 31-37.

Nagai,T. (1987). Parthenogenetic activation of cattle follicular oocytes in vitro with ethanol. Gamete Res *16*, 243-249.

Nagai,T., Ding,J., and Moor,R.M. (1993). Effect of follicle cells and steroidogenesis on maturation and fertilization in vitro of pig oocytes. J Exp Zool *266*, 146-151.

Nagai,T. (1996). In vitro maturation and fertilization of pig oocytes. Anim Reprod Sci *42*, 153-163.

Naito,K., Fukuda,Y., and Toyoda,Y. (1988). Effects of porcine follicular fluid on male pronucleus formation in porcine oocytes matured in vitro. Gamete Res *21*, 289-295.

Naito,K., Daen,F.P., and Toyoda,Y. (1992). Comparison of histone H1 kinase activity during meiotic maturation between two types of porcine oocytes matured in different media in vitro. Biol Reprod *47*, 43-47.

Nussbaum,D.J. and Prather,R.S. (1995). Differential effects of protein synthesis inhibitors on porcine oocyte activation. Mol Reprod Dev *41*, 70-75.

Ocampo,M.B., Ocampo,L.C., Mori,T., Ueda,J., and Kanagawa,H. (1994). Nuclear and cytoplasmic maturation of pig oocytes cultured in the amniotic fluid of developing chick embryos. J Vet Med Sci *56*, 173-176.

Onishi,A., Iwamoto,M., Akita,T., Mikawa,S., Takeda,K., Awata,T., Hanada,H., and Perry,A.C. (2000). Pig cloning by microinjection of fetal fibroblast nuclei. Science *289*, 1188-1190.

Ono,Y., Shimozawa,N., Ito,M., and Kono,T. (2001). Cloned mice from fetal fibroblast cells arrested at metaphase by a serial nuclear transfer. Biol Reprod *64*, 44-50.

Ozil,J.P. (1990). The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation. Development *109*, 117-127.

Ozil,J.P. and Swann,K. (1995). Stimulation of repetitive calcium transients in mouse eggs. J Physiol *483 ( Pt 2)*, 331-346.

Palermo,G.D., Avrech,O.M., Colombero,L.T., Wu,H., Wolny,Y.M., Fissore,R.A., and Rosenwaks,Z. (1997). Human sperm cytosolic factor triggers Ca2+ oscillations and overcomes activation failure of mammalian oocytes. Mol Hum Reprod *3*, 367-374.

Parrington,J., Swann,K., Shevchenko,V.I., Sesay,A.K., and Lai,F.A. (1996). Calcium oscillations in mammalian eggs triggered by a soluble sperm protein. Nature *379*, 364-368.

Petr,J., Zetova,L., Fulka,J.J., and Jilek,F. (1989). Quantitative inhibitory influence of porcine cumulus cells upon the maturation of pig and cattle oocytes in vitro. Reprod Nutr Dev *29*, 541-549.

Petters,R.M. and Wells,K.D. (1993). Culture of pig embryos. J Reprod Fertil Suppl *48*, 61-73.

Pickering,S.J.B.P.R.J.M.H.C.A.C.J. (1990). Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. Fertil Steril. *54*, 102-108.

Polejaeva,I.A., Chen,S.H., Vaught,T.D., Page,R.L., Mullins,J., Ball,S., Dai,Y., Boone,J., Walker,S., Ayares,D.L., Colman,A., and Campbell,K.H. (2000). Cloned pigs produced by nuclear transfer from adult somatic cells. Nature *407*, 86-90.

Prather,R.S., Sims,M.M., and First,N.L. (1989). Nuclear transplantation in early pig embryos. Biol Reprod *41*, 414-418.

Prather,R.S. and First,N.L. (1990). Cloning of embryos. J Reprod Fertil Suppl *40*, 227-234.

Presicce,G.A. and Yang,X. (1994). Nuclear dynamics of parthenogenesis of bovine oocytes matured in vitro for 20 and 40 hours and activated with combined ethanol and cycloheximide treatment. Mol Reprod Dev *37*, 61-68.

Prochazka,R., Kanka,J., Sutovsky,P., Fulka,J., and Motlik,J. (1992). Development of pronuclei in pig oocytes activated by a single electric pulse. J Reprod Fertil *96*, 725-734.

Raz,T., Ben-Yosef,D., and Shalgi,R. (1998). Segregation of the pathways leading to cortical reaction and cell cycle activation in the rat egg. Biol Reprod *58*, 94-102.

Rideout,W.M., Wakayama,T., Wutz,A., Eggan,K., Jackson-Grusby,L., Dausman,J., Yanagimachi,R., and Jaenisch,R. (2000). Generation of mice from wild-type and targeted ES cells by nuclear cloning. Nat Genet *24*, 109-110.

Robl,J.M., Collas,P., Fissore,R., and Dorbrinsky,J. (1992). Electrically induced fusion and activation in nuclear transplant embryos. In Guide to Electroporation and Electrofusion, D.C.Chang, J.A.Saunders, B.M.Chassy, and A.E.Sowers, eds. Academic Press,), pp. 535-551.

Schnieke,A.E., Kind,A.J., Ritchie,W.A., Mycock,K., Scott,A.R., Ritchie,M., Wilmut,I., Colman,A., and Campbell,K.H. (1997). Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science *278*, 2130-2133.

Schultz,R.M. and Kopf,G.S. (1995). Molecular basis of mammalian egg activation. Curr Top Dev Biol *30*, 21-62.

Shevchenko V,H.M.E.R.P.J.L.F. (1998). The human glucosamine-6-phosphate deaminase gene: CDAN cloining and expression, genomic organization and chromosomal localization . Gene *216*, 31-38.

Shevchenko V,H.M.E.R.P.J.L.FA. (1998). The human glucosamine -6-phosphate deaminase gene: cDAN cloning and expression, genomic organization and chromosomal localization. Gene *216*, 38.

Shiina,Y., Kaneda,M., Matsuyama,K., Tanaka,K., Hiroi,M., and Doi,K. (1993). Role of the extracellular Ca2+ on the intracellular Ca2+ changes in fertilized and activated mouse oocytes. J Reprod Fertil *97*, 143-150.

Shilling,F.M., Carroll,D.J., Muslin,A.J., Escobedo,J.A., Williams,L.T., and Jaffe,L.A. (1994). Evidence for both tyrosine kinase and G-protein-coupled pathways leading to starfish egg activation. Dev Biol *162*, 590-599.

Smith,L.C. and Wilmut,I. (1989). Influence of nuclear and cytoplasmic activity on the development in vivo of sheep embryos after nuclear transplantation. Biol Reprod *40*, 1027-1035.

Snell,W.J. and White,J.M. (1996). The molecules of mammalian fertilization. Cell *85*, 629-637.

Solter,D. (2000). Mammalian cloning: advances and limitations. Nat Rev Genet *1*, 199-207.

Sophie Brind,K.S.a.J.C. (2000). Inositol 1,4,5,-Trisphosphate Receptors are Downregulated in Mouse Oocytes in Response to Sperm or Adenophostin A but

Not to Increases in Intracellular Ca2+ or Egg activation. Developmental Biology *223*, 251-265.

Sowers,A.E.a.L.M.R. (2001). Electropore diameter, lifetimes, numbers, and locations in individual erythrocyte ghosts. FEBS Lett. *205*, 179-184.

Spemann,H. (1938). Embryonic Induction. (New York: Hafner).

Steinhardt,R.A. and Epel,D. (1974). Activation of sea-urchin eggs by a calcium ionophore. Proc Natl Acad Sci U S A *71*, 1915-1919.

Stice,S.L. and Robl,J.M. (1990). Activation of mammalian oocytes by a factor obtained from rabbit sperm. Mol Reprod Dev *25*, 272-280.

Stice,S.L., Keefer,C.L., and Matthews,L. (1994). Bovine nuclear transfer embryos: oocyte activation prior to blastomere fusion. Mol Reprod Dev *38*, 61-68.

Stricker,S.A. (1996). Repetitive calcium waves induced by fertilization in the nemertean worm Cerebratulus lacteus. Dev Biol *176*, 243-263.

Sun,F.Z., Hoyland,J., Huang,X., Mason,W., and Moor,R.M. (1992). A comparison of intracellular changes in porcine eggs after fertilization and electroactivation. Development *115*, 947-956.

Surani,M.A., Barton,S.C., and Norris,M.L. (1984). Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. Nature *308*, 548-550.

Surani,M.A., Barton,S.C., and Norris,M.L. (1987). Influence of parental chromosomes on spatial specificity in androgenetic----parthenogenetic chimaeras in the mouse. Nature *326*, 395-397.

Surani,M.A. (1991). Influence of genome imprinting on gene expression, phenotypic variations and development. Hum Reprod *6*, 45-51.

Surani,M.A. (1998). Imprinting and the initiation of gene silencing in the germ line. Cell *93*, 309-312.

Swann,K. (1990). A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. Development *110*, 1295-1302.

Swann,K. (1992). Different triggers for calcium oscillations in mouse eggs involve a ryanodine-sensitive calcium store. Biochem J *287 ( Pt 1)*, 79-84.

Swann,K. (1994). Ca2+ oscillations and sensitization of Ca2+ release in unfertilized mouse eggs injected with a sperm factor. Cell Calcium *15*, 331-339.

Tanaka,H. and Kanagawa,H. (1997). Influence of combined activation treatments on the success of bovine nuclear transfer using young or aged oocytes. Anim Reprod Sci *49*, 113-123.

Tarkowski,A.K. (1975). Induced parthenogenesis in the mouse. Symp Soc Dev Biol 107-129.

Thomson,J.A., Itskovitz-Eldor,J., Shapiro,S.S., Waknitz,M.A., Swiergiel,J.J., Marshall,V.S., and Jones,J.M. (1998). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145-1147.

Tian Yow Tsong (1989). Electroporation of Cell Membranes (Mechanisms and Applications. Chapter 9 in Electroporation and Electrofusion in Cell Biology Edited by Neumann. E, Sowers. AE and Jordan CA.

Tombes,R.M., Simerly,C., Borisy,G.G., and Schatten,G. (1992). Meiosis, egg activation, and nuclear envelope breakdown are differentially reliant on Ca2+, whereas germinal vesicle breakdown is Ca2+ independent in the mouse oocyte. J Cell Biol *117*, 799-811.

Trounson,A. and Pera,M. (1998). Potential benefits of cell cloning for human medicine. Reprod Fertil Dev *10*, 121-125.

Varmuza,S., Mann,M., and Rogers,I. (1993). Site of action of imprinted genes revealed by phenotypic analysis of parthenogenetic embryos. Dev Genet *14*, 239-248.

Vatzias,G. and Hagen,D.R. (1999). Effects of porcine follicular fluid and oviduct-conditioned media on maturation and fertilization of porcine oocytes in vitro. Biol Reprod *60*, 42-48.

Vincent,C., Cheek,T.R., and Johnson,M.H. (1992). Cell cycle progression of parthenogenetically activated mouse oocytes to interphase is dependent on the level of internal calcium. J Cell Sci *103 ( Pt 2)*, 389-396.

Vitullo,A.D. and Ozil,J.P. (1992). Repetitive calcium stimuli drive meiotic resumption and pronuclear development during mouse oocyte activation. Dev Biol *151*, 128-136.

Wakayama,T., Perry,A.C., Zuccotti,M., Johnson,K.R., and Yanagimachi,R. (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature *394*, 369-374.

Wakayama,T., Rodriguez,I., Perry,A.C., Yanagimachi,R., and Mombaerts,P. (1999). Mice cloned from embryonic stem cells. Proc Natl Acad Sci U S A *96*, 14984-14989.

Wakayama,T. and Yanagimachi,R. (1999). Cloning of male mice from adult tail-tip cells. Nat Genet *22*, 127-128.

Wakayama,T., Shinkai,Y., Tamashiro,K.L., Niida,H., Blanchard,D.C., Blanchard,R.J., Ogura,A., Tanemura,K., Tachibana,M., Perry,A.C., Colgan,D.F., Mombaerts,P., and Yanagimachi,R. (2000). Cloning of mice to six generations. Nature *407*, 318-319.

Wang,W. and Niwa,K. (1995). Synergetic effects of epidermal growth factor and gonadotropins on the cytoplasmic maturation of pig oocytes in a serum-free medium. Zygote *3*, 345-350.

Wang,W.H., Niwa,K., and Okuda,K. (1991). In-vitro penetration of pig oocytes matured in culture by frozen-thawed ejaculated spermatozoa. J Reprod Fertil *93*, 491-496.

Wang,W.H., Abeydeera,L.R., Okuda,K., and Niwa,K. (1994). Penetration of porcine oocytes during maturation in vitro by cryopreserved, ejaculated spermatozoa. Biol Reprod *50*, 510-515.

Wang,W.H., Abeydeera,L.R., Cantley,T.C., and Day,B.N. (1997). Effects of oocyte maturation media on development of pig embryos produced by in vitro fertilization. J Reprod Fertil *111*, 101-108.

Wang,W.H., Machaty,Z., Abeydeera,L.R., Prather,R.S., and Day,B.N. (1998). Parthenogenetic activation of pig oocytes with calcium ionophore and the block to sperm penetration after activation. Biol Reprod *58*, 1357-1366.

Wang,W.H., Machaty,Z., Ruddock,N., Abeydeera,L.R., Boquest,A.C., Prather,R.S., and Day,B.N. (1999). Activation of porcine oocytes with calcium ionophore: effects of extracellular calcium. Mol Reprod Dev *53*, 99-107.

Ware,C.B., Barnes,F.L., Maiki-Laurila,M., and First,N.L. (1989). Age dependence of bovine oocyte activation. Gamete Res *22*, 265-275.

Weaver,J.C. (1994). Molecular basis for cell membrane electroporation. Ann NY Acad Sci *720*, 141-152.

Weaver,J. (1995). Electroporation Theory ( Concepts and mechanisms). Animal Cell Electroporation and Electrofusion protocols Edited by J. A. Nickoloff *Methds in Molecular Biology 48*, 3-28.

Wells,D.N., Misica,P.M., and Tervit,H.R. (1999). Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. Biol Reprod *60*, 996-1005.

Wells,K., Moore,K., and Wall,R. (1999). Transgene vectors go retro. Nat Biotechnol *17*, 25-26.

Westhusin,M.E., Pryor,J.H., and Bondioli,K.R. (1991). Nuclear transfer in the bovine embryo: a comparison of 5-day, 6-day, frozen-thawed, and nuclear transfer donor embryos. Mol Reprod Dev *28*, 119-123.

Westhusin,M.E., Long,C.R., Shin,T., Hill,J.R., Looney,C.R., Pryor,J.H., and Piedrahita,J.A. (2001). Cloning to reproduce desired genotypes. Theriogenology *55*, 35-49.

Whitaker,M. and Swann,K. (1993). Lighting the fuse at fertilization. Development *117*, 1-12.

Whittingham,D.G. (1980). Parthenogenesis in mammals. C.A.Finn, ed., pp. 205-229.

Willadsen,S.M. (1986). Nuclear transplantation in sheep embryos. Nature *320*, 63-65.

Williams,C.J., Schultz,R.M., and Kopf,G.S. (1992). Role of G proteins in mouse egg activation: stimulatory effects of acetylcholine on the ZP2 to ZP2f conversion and pronuclear formation in eggs expressing a functional m1 muscarinic receptor. Dev Biol *151*, 288-296.

Wilmut,I., Schnieke,A.E., McWhir,J., Kind,A.J., and Campbell,K.H. (1997). Viable offspring derived from fetal and adult mammalian cells. Nature *385*, 810-813.

Wilmut,I. and Campbell,K.H. (1998). Quiescence in nuclear transfer. Science *281*, 1611.

Wolosker H,K.D.B.Y.l.S.C.A.F.T.S.R.S.SH. (1998). Molecularly cloned mammalian glucosamine-6 phosphate deaminase localizes to the transporting epithelium and lacks oscillin activity. FASEB J *12*, 91-99.

Wu H,H.C.-L.J.B.B.S.F.R. (1998). Partial characterization of the aclcium- releasing activitity of porcine sperm cytosolic extracts. Dev Biol *203*, 369-381.

Wu,H., He,C.L., and Fissore,R.A. (1997). Injection of a porcine sperm factor triggers calcium oscillations in mouse oocytes and bovine eggs. Mol Reprod Dev *46*, 176-189.

Wu,H., He,C.L., and Fissore,R.A. (1998). Injection of a porcine sperm factor induces activation of mouse eggs. Mol Reprod Dev *49*, 37-47.

Yamauchi,N., Sasada,H., Sugawara,S., and Nagai,T. (1996). Effect of culture conditions on artificial activation of porcine oocytes matured in vitro. Reprod Fertil Dev *8*, 1153-1156.

Yang,X., Presicce,G.A., Moraghan,L., Jiang,S., and Foote,R.H. (1994). Synergistic effect of ethanol and cycloheximide on activation of freshly matured bovine oocytes. Theriogenology *41*, 395-403.

Yim,D.L., Opresko,L.K., Wiley,H.S., and Nuccitelli,R. (1994). Highly polarized EGF receptor tyrosine kinase activity initiates egg activation in Xenopus. Dev Biol *162*, 41-55.

Yoshida,M., Ishizaki,Y., and Kawagishi,H. (1990). Blastocyst formation by pig embryos resulting from in-vitro fertilization of oocytes matured in vitro. J Reprod Fertil *88*, 1-8.

Yoshida,M., Ishizaki,Y., Kawagishi,H., Bamba,K., and Kojima,Y. (1992). Effects of pig follicular fluid on maturation of pig oocytes in vitro and on their subsequent fertilizing and developmental capacity in vitro. J Reprod Fertil *95*, 481-488.

Yoshida,M., Ishigaki,K., Nagai,T., Chikyu,M., and Pursel,V.G. (1993). Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. Biol Reprod *49*, 89-94.

Zawada,W.M., Cibelli,J.B., Choi,P.K., Clarkson,E.D., Golueke,P.J., Witta,S.E., Bell,K.P., Kane,J., Ponce,d.L.F., Jerry,D.J., Robl,J.M., Freed,C.R., and Stice,S.L. (1998). Somatic cell cloned transgenic bovine neurons for transplantation in parkinsonian rats. Nat Med *4*, 569-574.

Zhu,J., Harkness,L., King,T., Fletcher,J., Telfer,E.E., Wilmut,I., and De Sousa,P. (2000). Fetal development from parthenogenetically activated in vitro matured porcine oocytes. J Reprod Fertil *Abstract series 25*, 174.

Zimmermann,U., Vienken,J., and Pilwat,G. (1982). Development of drug carrier systems. J Electroanal Chem *116*, 553-574.

# Appendix-I

## IVM PROCEDURE (PIG)

*NCSU 23 medium (North Carolina State University)*

| Component | | Mol wt | Conc. (mM) | g/1000ml | g/500ml |
|---|---|---|---|---|---|
| NaCl | (Fisher S271) | 58.45 | 108.73 | 6.3553 | 3.1776 |
| $NaHCO_3$ | (Sigma S5761) | 84.00 | 25.07 | 2.1059 | 1.0529 |
| KCl | (Sigma S4505) | 74.55 | 4.78 | 0.3563 | 0.1782 |
| $KH_2PO_4$ | (Fisher P285) | 136.09 | 1.19 | 0.1619 | 0.0810 |
| $MgSO_4 \cdot 7H_2O$ | (Sigma M1880) | 246.50 | 1.19 | 0.2933 | 0.1467 |
| $CaCl_2 \cdot 2H_2O$ | (Sigma C7902) | 147.00 | 1.70 | 0.2499 | 0.1250 |
| Glucose | (Sigma G7021) | 180.20 | 5.55 | 1.0001 | 0.5001 |
| Glutamine | (Sigma G5763) | 146.10 | 1.00 | 0.1461 | 0.0731 |
| Taurine | (Sigma T7146) | 125.10 | 7.00 | 0.8757 | 0.4379 |
| Hypotaurine | (Sigma H1384) | 109.10 | 5.00 | 0.5455 | 0.2728 |
| Cysteine | (Sigma C8152) | 157.60 | 1.00 | 0.1576 | 0.0788 |
| Insulin | (Sigma I6634) | - | 5 mg/l | 0.0050 | 0.0025 |
| Penicillin G | (Sigma PEN-K) | - | 100iu/ml | (0.0650) | (0.0325) |
| Streptomycin | (Sigma S6501) | - | 50mg/l | 0.0500 | 0.0250 |

pH 7.4

Before preparation of maturation medium, add 1.00-mM cysteine, 10% pig follicular fluid and hormones, also add BME amino acids solution 50x (SIGMA B-6766.100ml/bottle) 0.2 ml/10 ml. and MEM non-essential amino acids solution 100x(SIGMA-M-7145, 100ml/bottle) 0.1ml /10ml.to the maturation medium.

A filter (0.22μm) with vacuum filter system (Coring #25942-500).

Store at 4°C and use within 2 weeks.

# Appendix-II

***TL-HEPES-PVA***

| Component | Mol wt | Conc. (mM) | g/L | g/2L |
|---|---|---|---|---|
| NaCl | 58.45 | 114.00 | 6.6633 | 13.3266 |
| KCl | 74.55 | 3.20 | 0.2386 | 0.4772 |
| $NaHCO_3$ | 84.00 | 2.00 | 0.1680 | 0.3360 |
| $NaH_2PO_4$ | 120.00 | 0.34 | 0.0408 | 0.0816 |
| Na-lactate* | 112.10 | 10.00 | 1.868 ml | 3.736 ml |
| $MgCl_2 \cdot 6H_2O$ | 203.30 | 0.50 | 0.1017 | 0.2034 |
| HEPES | 238.30 | 10.00 | 2.3830 | 4.7660 |
| $CaCl_2 \cdot 2H_2O$ | 147.00 | 2.00 | 0.2940 | 0.5880 |
| Na pyruvate | 110.00 | 0.19 | 0.0220 | 0.0440 |
| Penicillin G | - | 100 IU/ml | 0.0650 | 0.1300 |
| Gentamycin | - | 25μg/ml | 0.0250 | 0.0500 |
| D- Glucose | | | 0.900 | 1.8000 |
| PVA | - | - | 0.1000 | 0.2000 |

Adjust pH to 7.4.

60% syrup.

** should be added slowly to prevent formation of precipitates.

# Appendix-III

***Hepes – buffered-NCSU 23 medium (North Carolina State University)***

| Component | | Mol wt | Conc. (mM) | g/1000ml | g/500ml |
|---|---|---|---|---|---|
| NaCl | (Fisher S271) | 58.45 | 108.73 | 6.3553 | 3.1776 |
| $NaHCO_3$ | (Sigma S5761) | 84.00 | 2.0 | 0.168 | 0.084 |
| KCl | (Sigma S4505) | 74.55 | 4.78 | 0.3563 | 0.1782 |
| $KH_2PO_4$ | (Fisher P285) | 136.09 | 1.19 | 0.1619 | 0.0810 |
| $MgSO_4 \cdot 7H_2O$ | (Sigma M1880) | 246.50 | 1.19 | 0.2933 | 0.1467 |
| $CaCl_2 \cdot 2H_2O$ | (Sigma C7902) | 147.00 | 1.70 | 0.2499 | 0.1250 |
| Glucose | (Sigma G7021) | 180.20 | 5.55 | 1.0001 | 0.5001 |
| Glutamine | (Sigma G5763) | 146.10 | 1.00 | 0.1461 | 0.0731 |
| Taurine | (Sigma T7146) | 125.10 | 7.00 | 0.8757 | 0.4379 |
| Hypotaurine | (Sigma H1384) | 109.10 | 5.00 | 0.5455 | 0.2728 |
| Cysteine | (Sigma C8152) | 157.60 | 1.00 | 0.1576 | 0.0788 |
| Insulin | (Sigma I6634) | - | 5 mg/l | 0.0050 | 0.0025 |
| Penicillin G | (Sigma PEN-K) | - | 100iu/ml | (0.0650) | (0.0325) |
| Streptomycin | (Sigma S6501) | - | 50mg/l | 0.0500 | 0.0250 |
| Hepes | (Sigma H-9136) | 238.30 | 10.0 | 2.383 | 1.1915 |

pH 7.4

$CaCl_2 \cdot 2H_2O$ should be added into the medium after others have been resolved completely

# Appendix- IV

## *Activation medium*

| Composition | Catalogue No. | Mol. Wt | Conc. (mM) | g/1000ml |
|---|---|---|---|---|
| D-Mannitol | | 182.2 | 300 | 54.66 |
| $MgSO_4 \cdot 7H_2O$ | (Sigma M1880) | 246.5 | 0.1 | 0.2465 |
| $CaCl_2 \cdot 2H_2O$ | (Sigma C7902) | 147.00 | 0.05 | 0.0735 |

Dissolve in Millipore water and adjust osmolarity to 280 mOsmol. Filter and freeze in 1-ml aliquots at – 20°C.

Note, D- Sorbitol (S-1876 Sigma, FW 182.2) can replace D-Mannitol.

# Appendix-V

## *Zimmermann Cell Fusion Medium*

| Component | Sigma # | Mol wt | G/l | Concentration |
|---|---|---|---|---|
| Sucrose | S-9378 | 342.3 | 95.84 | 280 m*M* |
| Mg acetate.4 $H_2O$ | M-0631 | 214.5 | 0.016 | 0.5 m*M* |
| Ca acetate | C-1000 | 158.2 | 0.016 | 0.1 m*M* |
| $K_2PO_4$, anhydrous | P-8281 | 174.2 | 0.174 | 1.0 m*M* |
| Glutathione | G-4251 | 307.3 | 0.031 | 0.1 m*M* |
| BSA-FAF-Elisa grade | H-7030 | 25.000 | 0.01 | 0.01 mg/mL |

Adjust pH to 7.0 using 1N acetic acid. If pH is below 7, throw out and start again.

# Appendix-VI

**Fetal development from parthenogenetically activated *in vitro* matured porcine oocytes**
***Journal of Reproduction and Fertility* 2000 (25), Abstr 147.**

J Zhu[1], L Harkness[1], T King[1], J Fletcher[1], E Telfer[2], I Wilmut[1] & P De Sousa[1].
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Although a broad range of physical and chemical stimuli activate eggs, not all are equally capable of effectively mimicking the early events of fertilization and inducing development beyond the blastocyst stage. In the present study our objective was to assess the quality and competence of *in vitro* matured (IVM) porcine oocytes activated with a previously optimized electrical stimulus protocol. IVM oocytes were activated 44 h post maturation by first being given a 5 sec pulse of 0.25kV/cm AC followed by 3x80μs pulses of 1.0 kV/cm DC in 0.3 M Mannitol, 0.1mM $Mg^{2+}$, 0.05 M $Ca^{2+}$. Activated oocytes were then cultured in NCSU-23 + 7.5μg/ml cytochalasin-b for 6h to suppress polar body emission and restore a diploid karyotype. Development to the blastocyst stage was obtained by culturing in NCSU-23 for 6 days at 39°C, in 5%$CO_2$ in air. The ability to form a fetus on 21 days of gestation was assessed by transferring 60 parthenogenetic embryos per recipient 1 day post activation. As a control for normalcy, fertilized embryos collected 2 days post-oestrus were either maintained in culture for 5 days, or transferred within 3 hr to a final recipient (20 per gilt). Parthenogeneticaly activated IVM porcine oocytes were predominantly diploid (N=73, 83.6% 2n, 0% 1n, 16.4% 3n or greater), and were not significantly different from fertilized embryos in blastocyst rate (Parth vs Fert: 41.42+/-15.61($n_{=4}$) vs 75.33+/-25.37 (n=3), $p>0.05$) and nuclear count (Parth vs Fert: 26.62+/-10.31 (n=45) vs 33.53+/-11.77(n=17), $p>0.05$). Although the morphological stage of development was not different, fetuses developing from embryos were heavier (Fert vs Parth: 132.63+/-71.49gm (n=35) vs 57.36+/-16.92 (n=16), $p<0.01$), and longer (Fert vs Parth: 10.19+/-2.15mm (n=35) vs 7.9+/-1.06 (n=16), $p<0.01$). These results indicate that the defined activation protocol is capable of yielding advanced fetal development, and thus may be suitable for activation of cloned embryos following nuclear transfer.

# Appendix-VII

**Improving efficiency of *in vitro* matured pig oocyte activation**
***Theriogenology 2000, 53: Abst. 445***

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Although pig oocytes can be parthenogenetically activated, the rate of blastocyst formation is low. Four experiments were carried out to improve pig oocyte activation. After 37h of *in vitro* maturation (IVM), over 90% of oocytes were MII. Before oocyte activation, oocytes were aligned using a 5 sec pulse of 0.25kV/cm AC. Activated oocytes were cultured in NCSU-23 + 7.5μg/mL cytochalasin-B for 6h, and then NCSU-23 for 6-7 days at 39°C, in 5%$CO_2$ in air. In experiment 1, oocyte age and activation field strength were examined. Oocytes were activated at 36, 40 and 44h, with 1x80μsec. pulse of 1.0, 1.25 or 1.5kV/cm DC, respectively in 0.3 M Mannitol, 0.1mM $Mg^{2+}$, 0.05 mM $Ca^{2+}$. At d6, 44h IVM oocytes activated with 1 pulse of 1.5kV/cm produced the most blastocysts (41%, $p<0.05$). Experiments 2-4 used 44h IVM oocytes. In experiment 2, oocytes were activated with multiple pulses (1, 3 or 5) using field strengths and medium described above. Oocytes activated by 3x80 μsec pulses of 1.0 kV/cm yielded more blastocysts (54%, $p<0.01$) than in other treatments (<42%). In experiment 3, the benefit of $Ca^{2+}$ in the activation medium was tested. Activation media were (A) 0.3M Mannitol; (B) A + 0.1mM $Mg^{2+}$; (C) B + 0.05mM $Ca^{2+}$; (D) B + 0.1mM $Ca^{2+}$. Oocytes were activated by 3x80 μsec pulses of 1.0kV/cm DC and cultured to d7. Medium C gave the higher rate of blastocysts (44%, $p<0.05$). In experiment 4, pig oocytes were activated by 3x80 μsec pulses, 1.25kV/cm, in medium C, 0.3M Sorbitol, 0.1mM $Mg^{2+}$, 0.05mM $Ca^{2+}$, or Zimmerman's fusion medium. At d7, the blastocyst rate and mean cell number per blastocyst were not significantly different ($p>0.05$) between the 3 media (Mannitol: 36%, n=374, 45±16 cells; Sorbitol: 35%, n=381, 52± 21 cells; Zimmerman's: 41%, n=357, 46±21 cells).
Our data indicate that IVM pig oocytes activate best at 44h IVM. All 3 activation media proved to be equivalent in their ability to initiate parthenote development. The most effective protocol was a preliminary 0.25kV/cm AC pulse for 5 sec, followed by 3x80μsec pulses of 1.0 kV/cm DC

# Appendix-VIII

**Pig oocyte activation: The effect of maturation medium and activation condition**
***Theriogenology* 2001 (55): Abst. 459.**

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The objective of these experiments was to improve maturation medium and activation conditions for pig oocytes. In experiment1, pig oocytes were cultured in (1) NCSU 23 medium + 10% pig follicular fluid; (2) medium (1) + amino acids (AA); (3) medium (1) + 10ng/mL EGF and (4) medium (2) + 10ng/mL EGF, respectively. They were cultured in these media supplemented with 10IU/mL eCG and 10IU/Ml hCG at 39°C, 5%$CO_2$ in air for 22 h, then without hormonal supplements for additional 22 h. All the oocytes were activated in 0.3M Mannitol + 0.1mM $Mg^{2+}$ + 0.05mM $Ca^{2+}$ with 3x80μsec.pulses of 1.25kV/cm DC following a 5 sec. pulse of 0.25kV/cm AC. They were cultured in NCSU23 + 0.4% BSA + 7.5μg/mL cytochalasin B for 6 h, then cultured in NCSU23 medium + 0.4% BSA at 39°C, 5%$CO_2$ in air for 6 d. The experimental design and results show in Table 1.

In experiment 2, the time of oocyte maturation to MII was determined. A total of 436 oocytes were matured in medium 2. Samples were fixed in methanol: acetic acid (3:1) at 0, 22, 36, 37, 38, 39, 40, 41, 42, and 43 h of maturation, respectively, and stained with 1% orcein after 3-d fixation. 82 % oocytes at 0 h were at the GV stage, 68% oocytes at 22 h were at the MI stage. The percentage of MII oocytes was 75% at 36 h, over 90% from 37 to 43 h.

In experiment 3, a comparison was made of temperature being either 37°C on a warm stage or at room temperature (25°C) during the process of cumulus removing and oocyte activation. Oocytes were matured in medium (2) for 44 h. Denuded oocytes were activated in the activation medium within 30min. Activation was induced by 3x80μsec. pulses of 1.0 kV/cm DC following 0.25kV/cm AC for 5 sec. After 6h cytochalasin B treatment, they were cultured for 7 d. Blastocyst rate of oocytes activated at 37°C was significantly higher than that at 25°C (43.7%: 149 blastocysts/341oocytes vs. 32.4%: 120 /371; T- test, $p<0.01$).

Our data showed that addition of amino acids to maturation medium enhances development of activated porcine oocytes and that pig oocytes are sensitive to room temperature at activation. In these experimental conditions, there was no beneficial effect of including EGF in the maturation medium

**Table1**. Effect of amino acids and EGF in maturation medium

| Maturation medium | No.of oocytes (N) | No. of cleaved on D2 (N) | No. of blastocysts (N) | (%) | No. of nuclei /blastocyst Mean ± SD |
|---|---|---|---|---|---|
| NCSU 23 | 233 | 172 | 61 | 26.2 | 23.6 ±3.4 |
| NCSU23+EGF | 263 | 206 | 53 | 20.2[b] | 25.8 ± 5.0 |
| NCSU23+AA | 249 | 210 | 80 | 32.1[a] | 26.0 ± 4.4 |
| NCSU23+AA+EGF | 231 | 202 | 59 | 25.5[b] | 25.7 ± 4.9 |

There is a significant difference between *a* and *b* (T- test, $p < 0.05$).

The data were collected from 5 replicates.

# Appendix-IX:

**Pig cloning by somatic cell nuclear transfer**
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To clone a pig from somatic cells we first confirmed an electrical activation method on ovulated oocytes. We then evaluated delayed vs. simultaneous activation (DA vs. SA) strategies, 2 nuclear donor cells, and ovulated vs. *in vitro* matured oocytes as cytoplasts. Using enucleated ovulated oocytes as cytoplasts for fetal fibroblast nuclei and transferring cloned embryos into a recipient within 2 h of activation, a 2 h delay between electrical fusion and activation yielded blastocysts more reliably and with a higher nuclear count than SA. Comparable rate of development using DA were obtained following culture of embryos cloned from ovulated or *in vitro* matured cytoplasts and fibroblast or cumulus nuclei. Treatment of cloned embryos with cytochalasin B (CB) post fusion and for 6 h after DA had no impact on blastocyst development as compared with CB treatment post-fusion only. Using fetal fibroblasts as nuclear donor cells, a ive cloned piglet was produced in a pregnancy that was maintained by co-transfer of parthenogenetic embryos.